YEAST DNA-DEPENDENT RNA POLYMERASES

by

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ABSTRACT

Yeast DNA-Dependent RNA Polymerases

by Graeme I. Bell

Yeast DNA-dependent RNA polymerases I, II and III are phosphorylated *in vivo*. Yeast cells were grown continuously in $^{32}P_i$ and the RNA polymerases were isolated by a new procedure which allows the simultaneous purification of these enzymes. from small quantities (35-60 g) of cells. Each of the RNA polymerases was phosphorylated. The following phosphorylated polymerase polypeptides were identified: polymerase I subunits of 185,000, 44,000, 36,000, 24,000 and 20,000 daltons; a polymerase II subunit of 24,000 daltons; and polymerase III subunits of 24,000 and 20,000 daltons. The incorporated ^{32}P was acid stable but base labile. Phosphoserine and phosphothreonine were identified after partial acid hydrolysis of purified ^{32}P-polymerase I.

A yeast protein kinase that copurifies with polymerase I during part of the isolation procedure was partially purified and characterized. This protein kinase phosphorylates the subunits of the purified polymerases that are phosphorylated *in vivo* and in addition a polymerase I subunit of 48,000 daltons and a polymerase II subunit of 33,500 daltons. Phosphorylation of the purified enzymes with this protein kinase had no substantial effect on polymerase activity in simple assays using native yeast DNA as a template. Preincubation of purified polymerase I with acid or alkaline phosphatase also had no detectable effect on polymerase activity.

INTRODUCTION

The importance of gene expression in controlling growth and development now seems well established (1). In bacteria, many of the transcriptive components have been identified and their role in the regulation of gene expression established by a combination of genetic and biochemical techniques (2). In eucaryotes, studies of the regulation of gene expression at the molecular level have not achieved the same degree of sophistication as those in procaryotes because of the difficulty in isolating the various components, which are often in very low concentration, and the structural complexity of the template. However, it appears that the regulation of gene expression is more complex in eucaryotes than procaryotes (3).

As an experimental approach to the study of transcription in eucaryotes, we have chosen to attempt to reconstruct transcriptive specificity using purified components. This requires the isolation of the molecules involved in transcription and the development of assays to measure transcription of specific genes. Identifying and purifying the components of the transcriptive machinery and determining their role in the transcriptive process is a necessary first step in this approach.

In eucaryotes, there are multiple forms of DNA-dependent RNA polymerase (4). These enzymes, designated I, II and III by Roeder and Rutter, can be distinguished by their unique polypeptide structures, ion-exchange chromatographic and general enzymatic properties, and sensitivity to the inhibitor, α-amanitin (reviewed in ref. 5). Each enzyme form appears to have a different functional role in the cell.

For example, polymerase I synthesizes the large ribosomal RNA precursor, polymerase II synthesizes messenger RNA and polymerase III synthesizes 5S ribosomal and transfer RNAs. The possibility that polymerases I and III also synthesize other RNAs including messenger RNA has not been rigorously excluded though. Nevertheless, the multiplicity of RNA polymerases, each with apparently different transcriptive specificity, facilitates independent regulation of the synthesis of at least three classes of RNA.

Since 1969, when Roeder and Rutter first described the presence of multiple RNA polymerases, much of the research directed towards an understanding of the molecular biology of RNA synthesis in eucaryotes has concentrated on an analysis of transcription in vertebrates. Although it has been difficult to purify all three enzymes from the same source, small quantities of several enzyme forms, particularly polymerases I and II, have been purified from a number of vertebrate sources and their polypeptide composition determined (5,6). Studies in vertebrate systems established the functional roles of the polymerases and their apparent independent regulation. However, as investigators have shifted from an analysis of the polypeptide structure of these enzymes to cell-free studies of transcription using purified components and more detailed studies of the enzymes themselves, it became obvious that vertebrates were not ideal experimental systems. Although one or more of the forms of polymerase have been isolated from several vertebrate sources, it has been difficult to isolate all enzymes, especially polymerase III, from the same source. In fact, the purification of all three RNA polymerases has been accomplished from only one vertebrate source, a mouse myeloma

(7-9). Since it is difficult to obtain large quantities of starting material for the enzyme isolations, purification of sufficient quantities of the enzymes for detailed chemical or biochemical studies and transcription experiments is difficult. Besides being a poor source for RNA polymerases, vertebrates have another disadvantage, that is their genomic complexity. As a consequence, a gene present once per haploid vertebrate genome represents approximately 1×10^{-4}% of the DNA sequences. Genes present in multiple copies, for example the histone (10) and ribosomal (11) RNA genes, represent a larger percentage of the DNA sequences, but in the case of the large ribosomal RNA genes which are repeated approximately 1000 fold this still represents only 0.1% of the DNA sequences. In contrast, in bacteria and a lower eucaryote, yeast, a unique gene represents 0.02% and 0.01%, respectively, of the DNA sequences. The large ribosomal RNA genes in bacteria and yeast comprise approximately 1% and 2.5%, respectively, of the genome. In order to measure transcription of specific genes in vertebrates, both qualitatively and quantitatively, it is necessary to synthesize large quantities of RNA which in turn requires large quantities of purified transcriptive components. The molecular cloning (12) of fragments of vertebrate DNA containing specific genes, which can be used as templates for the purified enzymes, will help to overcome this problem but with the present restrictions on the cloning of vertebrate genes, the development of these templates is difficult.

In order to circumvent the problems associated with studying transcription in higher eucaryotes, we have chosen to develop the yeast, Saccharomyces cerevisiae, as a model eucaryotic system in which to study

the structural and transcriptive properties of RNA polymerases I, II and III. Yeast is a unicellular lower eucaryote. It can be obtained in large quantities commercially, at little cost, so that it should be possible to obtain sufficient quantities of the purified transcriptive components. The haploid yeast genome contains $6.5\text{-}9.2 \times 10^9$ daltons of DNA most of which reassociates with kinetics expected for unique sequences (3,13). The complexity of the yeast genome is approximately three times that of the bacterium, E. coli. Thus a unique gene is present at a concentration which is at least 100 times greater than of a unique gene in a vertebrate genome, and consequently its transcription is easier to measure. The lower genetic complexity will also facilitate the isolation of recombinant DNA molecules containing specific yeast genes which can be used as templates for the purified enzymes. Roeder and Rutter (14) had shown in their initial studies that yeast, like higher eucaryotes, possessed multiple forms of RNA polymerase. These studies were extended by others who demonstrated that the yeast enzymes possess chromatographic and general enzymatic properties similar to their cognate enzymes in vertebrates (15,16). Yeast chromatin has been isolated and characterized (17,18). The DNA is associated with the four core histones, H2A, H2B, H3 and H4, in nucleosomes. Histone H1 appears to be absent from yeast chromatin but has been found in other lower eucaryotes, Neurospora (19) and Aspergillus (20), and more careful analysis will probably establish its presence in yeast as well. The nucleosomes appear more closely packed in yeast chromatin than they are in chromatin isolated from higher eucaryotes but the size of the DNA protected against nuclease digestion in the nucleosome is the same (18,21). Thus, yeast has the

genomic complexity of a procaryote, but the multiple RNA polymerases and chromatin structure characteristic of higher eucaryotes.

Yeast also has several other properties which make it attractive as a model transcriptive system. The multiple ribosomal RNA genes of yeast are present in gamma-DNA (22,23), a fraction representing approximately 10% of the nuclear DNA, and like those of Xenopus (11) can be isolated from the remaining non-ribosomal DNA sequences. The isolated ribosomal RNA genes provide a convenient assay for ribosomal gene transcription (24). The 5S ribosomal RNA genes of yeast are interspersed between the large ribosomal RNA genes (25), as they are bacteria (26). The 5S and large ribosomal RNAs do not appear to be synthesized via a common precursor in yeast though (27), as they are in bacteria (26). In fact, in yeast they appear to be synthesized from opposite strands of the DNA (28). The biosynthesis of stable RNA species has been well studied in yeast (27,29) and thus provides a useful foundation for in vitro studies of their synthesis. The primary structure of yeast 5.8S and 5S ribosomal RNAs as well as a number of transfer RNAs (32) has been determined. It will thus be possible to ask questions about the correct initiation and termination of transcription of the genes for 5S and transfer RNAs in chromatin and naked DNA by the purified RNA polymerases by combining hybridization and sequencing techniques, especially in the case of 5S ribosomal RNA which is apparently not synthesized as part of a precursor in vivo (23). The genetics of yeast are also well understood (33) and a large number of mutants defective in RNA synthesis are available (34,35), so that it might be possible to use both genetic and biochemical techniques to study transcription, as has been done in bacteria.

As a first step in developing yeast as a model system, it seemed necessary to isolate and characterize the three yeast RNA polymerases. This dissertation describes novel procedures developed for the simultaneous purification of RNA polymerases I, II and III from yeast and their application in a study of the modification of RNA polymerase by phosphorylation, as well as some properties of the isolated enzymes.

The activity of RNA polymerase appears to be modulated in many instances, but the regulatory mechanism(s) is unknown (5). In general, many studies have established that chemical modification of enzymes is one means of regulating activity (36). Chemical modification can result in increased or decreased enzyme activity, enzyme activation or inactivation, or a change in the catalytic properties of the enzyme. The regulation of enzyme activity by phosphorylation has been well documented, particularly in the regulation of glycogen metabolism. The phosphorylation of chromosomal components has also been reported and it has been suggested that phosphorylation of histones may be one means of altering the state of coiling or condensation of the chromatin (37) and that the phosphorylation of acidic proteins may be involved in gene activation (38). Consequently, it seemed reasonable that RNA polymerase activity might also be regulated by phosphorylation. On the basis of preliminary experiments, several groups (39-41), including ourselves (42), suggested that polymerase activity was regulated in this manner but were unable to convincingly demonstrate phosphorylation of polymerase polypeptides. Using the purification procedures described here, we have been able to demonstrate that yeast RNA polymerases I, II and III are phosphorylated *in vivo*. This analysis of polymerase modification demonstrated the utility of these enzyme purification procedures and as well

is the first conclusive demonstration that eucaryotic RNA polymerases can be chemically modified. We were unable, though, to show any substantial effect of phosphorylation on polymerase activity.

Now that the three yeast RNA polymerases have been purified in large quantities and characterized, it is possible to begin to test the functional properties of the isolated enzymes. It will be possible to determine whether the transcriptive specificity of the enzymes in vivo is present in the purified enzymes, that is, do the purified polymerases transcribe the appropriate sequences of purified yeast DNA, or are other chromosomal components required as well. It will be possible to begin to determine the function of each of the subunits of these structurally complex enzymes in the transcriptive process. Also, the purification procedures described here will allow the study of the synthesis and degradation of RNA polymerase and so elucidate the pathway by which the enzyme is assembled and the role of the cellular enzyme concentration in the regulation of RNA synthesis.

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PHOSPHORYLATION OF YEAST DNA-DEPENDENT RNA POLYMERASES *IN VIVO* AND *IN VITRO*. THE ISOLATION OF THE ENZYMES AND IDENTIFICATION OF THE PHOSPHORYLATED SUBUNITS

In eucaryotes, transcription is regulated during development and in response to physiological stimuli (reviewed in ref. 1,2). In principle, the intracellular function of RNA polymerase can be regulated by altering the enzyme concentration, by chromatin modifications which change the interaction between polymerase and template, or by modifications of the enzyme which alter its specific activity. It has been difficult to measure the relative contribution of each of these mechanisms in regulating transcription but, in a number of cases (3-8), the data have been interpreted to suggest that the increased polymerase activity may be due to a change in the rate of initiation or elongation of the enzyme rather than to a change in the absolute number of enzyme molecules. Several groups have reported that protein kinases stimulate the activity of polymerases I and II in cell-free systems (9-11) and suggest that RNA polymerase activity *in vivo* might be regulated through phosphorylation by protein kinases. However, these studies did not determine which (if any) of the component proteins were phosphorylated. In a preliminary report (12), we presented data which indicated that polymerase I was labeled when isolated from yeast cells grown in $^{32}P_i$ and that the yeast RNA polymerases I, II and III could be phosphorylated *in vitro* by a yeast protein kinase. This paper extends these studies and demonstrates that certain polypeptides of each of the three RNA polymerases are phosphorylated *in vivo* and also by a purified protein kinase *in vitro*. However, we have been unable to measure a significant effect of phosphorylation on the activity of these enzymes in conventional assays using native yeast DNA templates.

EXPERIMENTAL PROCEDURES

Yeast Strains. An auxotrophic tetraploid strain of Saccharomyces cerevisiae, 5178 $1C^2$ x $2B^2$, obtained from L. Hartwell, University of Washington, was used in all in vivo phosphate labeling experiments. Unlabeled RNA polymerases were isolated from a commercial strain of yeast, F1, a gift of the Red Star Yeast Co., Oakland, California. There were no apparent differences in the physical properties or the subunit composition of the enzymes isolated from these two sources.

Materials. Phosvitin, histone (calf thymus) and acid phosphatase (potato) were purchased from Calbiochem, La Jolla, California, Protamine-Cl, phosphoserine and phosphothreonine were obtained from Sigma Chemical Co., St. Louis, Missouri. Casein (Pentex) was purchased from Miles Laboratories Inc., Kankakee, Illinois and alkaline phosphatase (E. coli) from Worthington Biochemical Corp., Freehold, New Jersey. $^{32}P_i$ (as orthophosphoric acid in a 0.02 M HCl solution, carrier-free) was obtained from New England Nuclear, Boston, Massachusetts. A 50% solution of Polymin P was obtained from Gallard-Schlesinger Chem. Mfg. Corp., Carle Place, New York, diluted ten times to a 5% (w/v) solution, and the pH adjusted to 8.0 with HCl using pH indicator paper. Yeast DNA was isolated from the tetraploid strain grown in phosphate-depleted YEPD medium (13) by the procedure of Cryer et al. (14). This DNA had a double-stranded molecular weight of approximately 35×10^6 and was about half as efficient as commercial calf thymus DNA as a template for the purified yeast RNA polymerases. All other materials have been described previously (15).

Preparation of Resins and Buffers. Phosphocellulose, DEAE-cellulose and DEAE-Sephadex A-25 were precycled and equilibrated with the appropriate buffers as described previously (15). Denatured calf thymus DNA cellulose was prepared by the procedure of Alberts and Herrick (16). The buffers used are those described in Valenzuela et al. (15). Buffer A is 0.02 M Tris-HCl, pH 8.0, 0.01 M 2-mercaptoethanol, 0.5 mM EDTA and 10% glycerol. Buffer C is 0.02 M Tris-HCl, pH 8.0, 0.01 M 2-mercaptoethanol, 0.5 mM EDTA and 25% glycerol.

Growth Conditions and Preparation of Spheroplasts. Stationary phase cells were inoculated at a 1/100 dilution into phosphate-depleted YEPD medium containing 4-30 mCi/liter of $^{32}P_i$ and grown overnight at 30°. The generation time at mid-log phase of growth was approximately 150 minutes. The cells were harvested when the density reached approximately $8x10^7$ cells/ml and washed once with cold distilled water. Spheroplasts were prepared at 30° from the washed cells by the two step procedure of Cabib (17) as modified by Wintersberger et al. (18).

RNA Polymerase Assay. The standard incubation mixture of 0.050 ml contained 60 mM Tris-HCl, pH 7.9, 1.6 mM $MnCl_2$, 0.6 mM each of ATP, CTP and GTP, 0.01 mM [^{3}H]UTP (900 cpm/pmol), 10 mM 2-mercaptoethanol and 5 µg of heat-denatured calf thymus DNA. After incubation for 10 min at 30°, a 0.040 ml aliquot was applied to a Whatman DE81 filter disc. The filter discs were washed and counted as described previously (15).

Protein Kinase Assay. The standard incubation mixture of 0.050 ml contained 60 mM Tris-HCl, pH 7.9, 5 mM $MgCl_2$, 0.01 mM [γ-^{32}P]ATP (500-4000 dpm/pmol), 10 mM 2-mercaptoethanol and 0.20 mg of phosvitin. After incubation for 10 min at 30°, a 0.040 ml aliquot was applied to a

Whatman 3 MM filter disc. The discs were washed batchwise at 4° (6 times, 10 min each) in 10% trichloroacetic acid, 0.01 M sodium pyrophosphate and 0.01 M K_2HPO_4 to remove unincorporated isotope. The discs were then washed in ethanol, ether dried and counted in Omnifluor-toluene scintillant (New England Nuclear). When the specific activity of the different protein kinase fractions was measured, the ATP concentration was raised to 0.1 mM.

Polyacrylamide Gel Electrophoresis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was carried out in slabs as described previously (15). Buffers and solutions were prepared according to Laemmli (19). The polyacrylamide concentration of the resolving gel was either 10% or 11% as indicated.

Partial Acid Hydrolysis and Analysis of Hydrolyzate for Phosphoserine and Phosphothreonine. Thirty µg of purified ^{32}P-polymerase I (1500 dpm/µg) was precipitated from 5% trichloroacetic acid for 30 min at 0°. The sample was heated at 90° for 15 min to hydrolyze nucleic acids (20) and subsequently chilled at 0° for 30 min. The precipitate was collected by centrifugation (Sorvall HB4, 10,000g, 30 min, 0°), washed with ethanol: diethyl ether (1:1), acetone and then dried under N_2. The precipitate was dissolved in 0.2 ml of 5.7 N HCl (constant boiling) and hydrolyzed for 4 h at 110° as suggested by Bylund and Huang (21) for maximum recovery of phosphoserine. The hydrolyzate was evaporated to dryness in a vacuum dessicator over solid NaOH and phosphorus pentoxide. The residue was dissolved in 30 µl of electrophoresis buffer (2.5% formic acid, 7.8% acetic acid, pH 1.85) (20) containing 5 µg each of phosphoserine and phosphothreonine. The sample was applied to Whatman 3MM paper (22 inches long). Following electrophoresis at 4000 volts for 90 min, the

paper was dried and then sprayed with Cd-ninhydrin reagent (20). The radioactive compounds on the paper were located with respect to phosphoserine and phosphothreonine standards by autoradiography and these regions of the paper were cut out and counted in a liquid scintillation counter.

Enzyme Purification

DNA-Dependent RNA Polymerases. Unlabeled yeast RNA polymerases I, II and III were prepared as described elsewhere (15,22). ^{32}P-labeled polymerases I, II and III were purified simultaneously from spheroplasts by modifying the procedures described in detail by Valenzuela et al. (16,22). These modified procedures are presented below and describe the isolation of the three RNA polymerases from 35-60 g (wet weight) of yeast. All steps were carried out at 0-4°.

Preparation of Cell Extract. Yeast spheroplasts were collected from the spheroplast buffer (1 M sorbitol) by centrifugation (10 min at 27,000g). The pellet was suspended with a glass rod and finally a glass-Teflon homogenizer in 2 ml of extraction buffer (0.02 M Tris-HCl, pH 8.0, 10% glycerol, 0.5 mM EDTA, 0.01 M $MgCl_2$, 1 mM phenylmethylsulfonyl fluoride, 1% dimethyl sulfoxide and 1 mM ATP) per gram of cells. The viscous solution was sonicated (Branson sonicator) 6 x 10 sec at 100 watts output and the pH was adjusted to 8 by the addition of solid Tris base. The extract was then centrifuged 30 min, 48,000g (Sorvall SS34, 20,000 rpm) and the supernatant containing the RNA polymerase activity was treated as follows.

Purification of Polymerase I. RNA polymerase I was purified from the extract batchwise with phospho- and DEAE-cellulose to fraction 3

by the procedure of Valenzuela et al. (15). This procedure was reduced proportionately to accommodate the small amount of cells used. RNA polymerases II and III were purified from the protein not absorbed by phosphocellulose at 0.15 M ammonium sulfate in Buffer A. The polymerase I in fraction 3 was precipitated by dialysis against Buffer A saturated with ammonium sulfate. The precipitate was collected by centrifugation (27,000g, 30 min, Sorvall HB4), dissolved in 0.7 ml of 0.2 M KCl in Buffer A and layered onto a linear, 11.8 ml, 5-20% (w/v) sucrose gradient in 15% glycerol, 0.05 M Tris-HCl, pH 8.0, 0.5 mM EDTA, 0.5 M KCl and 10 mM 2-mercaptoethanol and centrifuged in a Beckman SW41 rotor at 40,000 rpm for 28 hr. The gradient fractions were assayed for RNA polymerase activity, protein concentration and ^{32}P-labeled protein. In measurements of ^{32}P-protein, nucleic acids were destroyed by heating the filters containing the sample in 5% TCA at 100° for 15 min (20). The polypeptide composition of fractions of the gradient was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Purification of Polymerase II. RNA polymerases II and III in the 0.15 M ammonium sulfate-Buffer A phosphocellulose filtrate were separated from the bulk of the nucleic acid and some of the protein by precipitation with and selective extraction from Polymin P. Eight ml of a 5% (w/v) solution of Polymin P was added dropwise per 100 ml of filtrate with stirring. After equilibration for 5 min, the suspension was centrifuged (27,000g, 20 min), the supernatant was discarded, and polymerases II and III were extracted from the pellet with 0.3 M ammonium sulfate in Buffer A containing 1 mM phenylmethylsulfonyl fluoride and 1% dimethyl sulfoxide (0.5 volumes relative to volume of original filtrate) by hand

homogenization. The suspension was centrifuged (27,000g, 20 min) and the Polymin P precipitate was discarded. Polymerases II and III were precipitated from the supernatant by the addition of solid ammonium sulfate (0.35 g/ml). The precipitate was collected by centrifugation (100,000g, 60 min) and dissolved in Buffer A. The ammonium sulfate concentration (as determined by conductivity measurements) was adjusted to 0.15 M with Buffer A. This solution was chromatographed on a DEAE-cellulose column (15 ml bed volume) equilibrated with 0.15 M ammonium sulfate in Buffer A. Under these conditions polymerase II is absorbed by the resin. The material which is not absorbed is processed for polymerase III as described below. The DEAE-cellulose column was washed with 0.15 M ammonium sulfate in Buffer A until the A_{280} of the effluent returned to background. The polymerase II was eluted with 0.3 M ammonium sulfate in Buffer A and then precipitated by dialysis against Buffer A containing 0.35 g/ml of ammonium sulfate. The precipitate was collected by centrifugation (27,000g, 30 min) and dissolved in sufficient Buffer A so that the ammonium sulfate concentration was less than 0.03 M. This solution was absorbed to a phosphocellulose column (6 ml bed volume) equilibrated with 0.03 M ammonium sulfate in Buffer A. After washing the column with this buffer, the polymerase was eluted with 0.14 M ammonium sulfate in Buffer A and then precipitated by dialysis against Buffer A containing 0.35 g/ml of ammonium sulfate. This precipitate was collected by centrifugation (27,000g, 30 min) and dissolved in 0.25 ml of 0.2 M KCl in Buffer A and layered onto a linear 5-20% (w/v) sucrose gradient that was prepared, sedimented and analyzed as described for polymerase I.

Purification of Polymerase III. The polymerase III in the flow-through from the DEAE-cellulose column was absorbed onto a DEAE-Sephadex A-25 column (15 ml bed volume) equilibrated with 0.15 M ammonium sulfate in Buffer A. After washing with 0.2 M ammonium sulfate in Buffer A. The polymerase fractions were pooled and the enzyme precipitated by dialysis against Buffer A containing 0.35 g/ml of ammonium sulfate. The precipitate was collected by centrifugation (27,000g, 30 min) and dissolved in sufficient Buffer C so that the conductivity was equivalent to 0.10 M KCl in Buffer C. This solution was absorbed to a denatured calf thymus DNA-cellulose column (5 ml bed volume, 100 µg DNA/ml packed cellulose) equilibrated with 0.10 M KCl in Buffer C. The column was washed with 0.15 M KCl in Buffer C and then the polymerase III was eluted with 0.70 M KCl in Buffer C. The polymerase III was precipitated by dialysis against Buffer A containing 0.35 g/ml of ammonium sulfate. The precipitate was collected by centrifugation (27,000g, 60 min), dissolved in 0.2 ml of 0.2 M KCl in Buffer A and layered on a linear, 4.6 ml, 5-20% (w/v) sucrose gradient in 0.2 M KCl in Buffer C. After sedimentation for 14 hr at 55,000 rpm (Beckman SW60), the gradient was analyzed as described for polymerase I.

Protein Kinase. RNA polymerase I, purified to fraction 4 by the procedure of Valenzuela *et al.* (15), was subjected to sucrose gradient sedimentation in 0.5 M KCl as described above. At salt concentrations less than 0.5 M KCl, the protein kinase activity is distributed throughout the sucrose gradient and contaminates polymerase I. The gradient was fractionated and assayed for protein kinase and RNA polymerase activity and protein concentration (Fig. 1). The protein kinase fractions

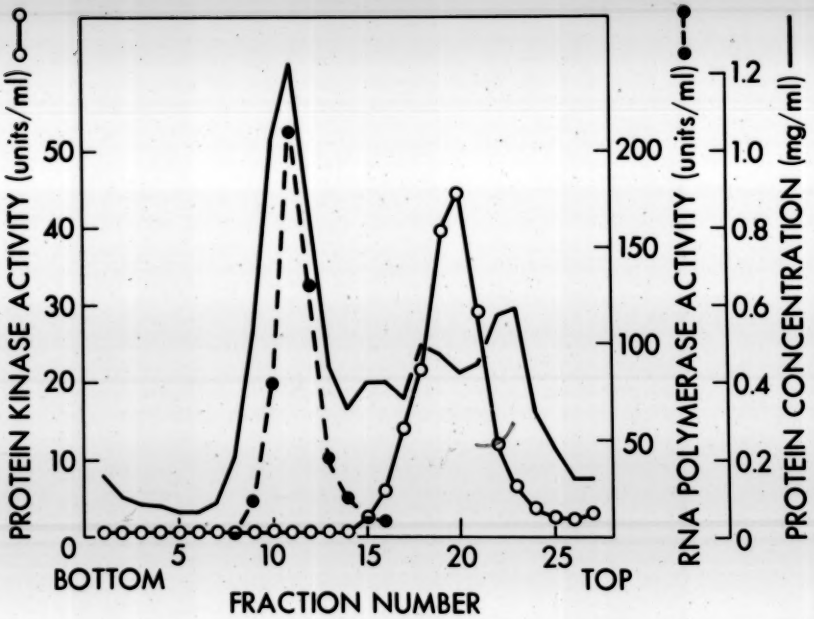


FIGURE 1. Sucrose density gradient centrifugation of yeast RNA polymerase I, fraction 4. Gradients were prepared, loaded and centrifuged as described in the text. Fractions were assayed for protein, RNA polymerase, and protein kinase activity.

were pooled, diluted with Buffer C to 0.2 M KCl and then absorbed to a phosphocellulose column (0.5 mg protein/ml of bed volume) equilibrated with 0.2 M KCl in Buffer C. The column was washed with 0.2 M KCl in Buffer C and then developed with a linear gradient from 0.2 M to 0.6 M KCl in Buffer C (10 column volumes) (Fig. 2). The protein kinase eluted at 0.38 M KCl. The enzyme fractions were pooled, dialyzed against 0.2 M KCl in Buffer C and then concentrated by absorption to a phosphocellulose column. The protein kinase was step eluted with 0.6 M KCl in Buffer C. Following dialysis against 0.05 M KCl in Buffer C, the kinase was absorbed to a column of DEAE-Sephadex A-25 (0.5 mg protein/ml of bed volume). The column was washed with 0.05 M KCl in Buffer C and then developed with a linear (10 column volumes) gradient of 0.05 M - 0.35 M KCl in Buffer C (Fig. 3). The protein kinase, eluting at 0.25 M KCl, was pooled and stored at -76°.

The protein kinase activity has been purified approximately 100-fold (Table I) from polymerase I, fraction 4. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis resolved the proteins in the DEAE-Sephadex pool into four major polypeptides with apparent molecular weights of 41,000, 38,000, 35,000 and 31,000 as well as several higher molecular weight polypeptides present in much lower amounts. The relationship of these polypeptides to the protein kinase activity has not been determined. The protein kinase (ion filtration pool) has a sedimentation coefficient ($S_{20,w}$) in 0.5-2.0 M KCl, as calculated by the procedure of Martin and Ames (23), of 6.8 relative to RNA polymerase I which has an $S_{20,w}$ of 16.2 (15). This suggests that the native protein kinase, if globular, has a molecular weight of approximately 135,000 and

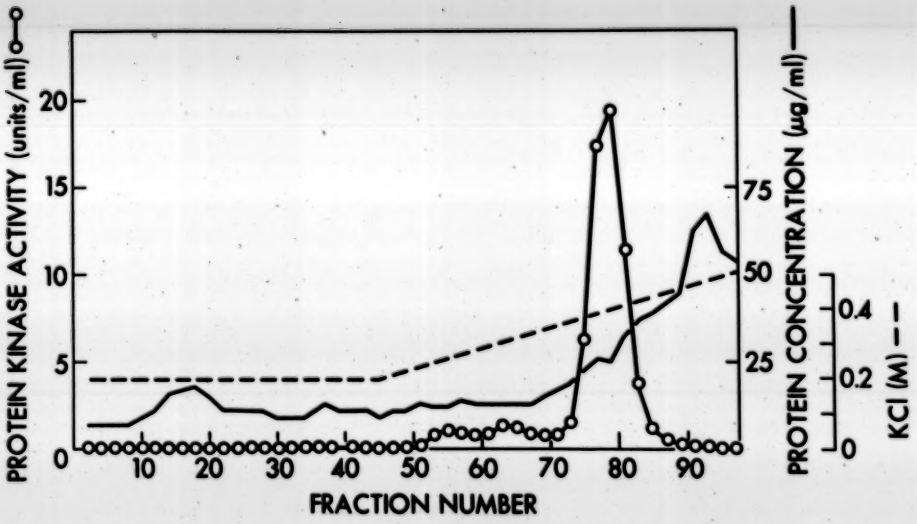


FIGURE 2. Phosphocellulose column chromatography of yeast protein kinase. The active fractions from the sucrose gradient were pooled (∿14 mg of protein), diluted to 0.2 M KCl with Buffer C and applied to a 28 ml phosphocellulose column (2.3 x 6.8 cm) equilibrated with 0.2 M KCl in Buffer C. The column was washed with 75 ml of 0.2 M KCl in Buffer C and then developed with a 300 ml linear gradient of 0.2 - 0.6 M KCl in Buffer C. 3.9 ml fractions were collected.

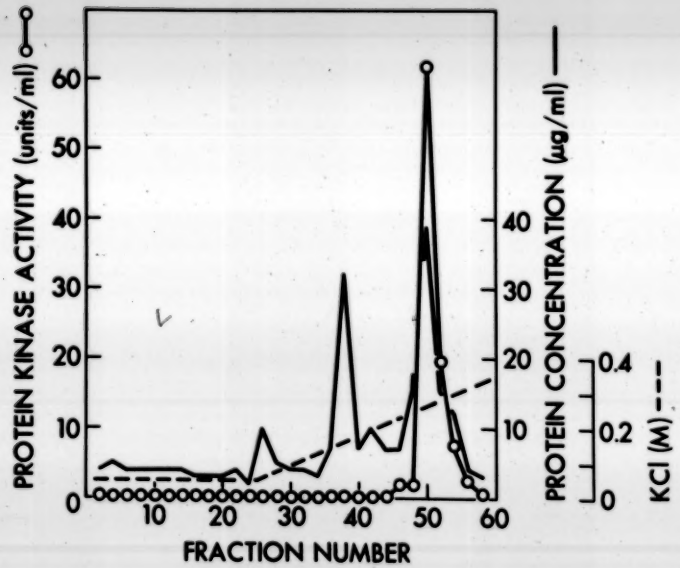


FIGURE 3. DEAE-Sephadex A-25 chromatography of yeast protein kinase. The concentrated protein kinase (∿200 µg) after phosphocellulose chromatography was dialyzed against 0.05 M KCl in Buffer C and then applied to 2.2 ml DEAE-Sephadex A-25 column (0.6 x 7.7 cm) equilibrated with 0.05 M KCl in Buffer C. After washing with 7 ml of 0.05 M KCl in Buffer C, the column was developed with a 22 ml linear gradient of 0.05 - 0.35 M KCl in Buffer C. 0.5 ml fractions were collected.

TABLE I

Summary of Purification of Yeast Protein Kinase

The data are taken from one representative experiment. Of wet weight of yeast cells, 2000 g were used as starting material. One unit corresponds to 1 nmol of ^{32}P incorporated in 10 min at 30° under the conditions described in experimental procedures.

Purification Step	Volume	Total Protein	Activity	Specific Activity	Yield
	ml	mg	units	units/mg	%
1. Ion filtration (DEAE-Sephadex)	106	70	7150	102	100
2. Sucrose gradient	22	13.6	6852	504	96
3. Phosphocellulose	34	0.68	2431	3574	34
4. Phosphocellulose -concentration	2	0.40	2700	6749	38
5. DEAE-Sephadex A-25	3.4	0.09	926	10,288	13

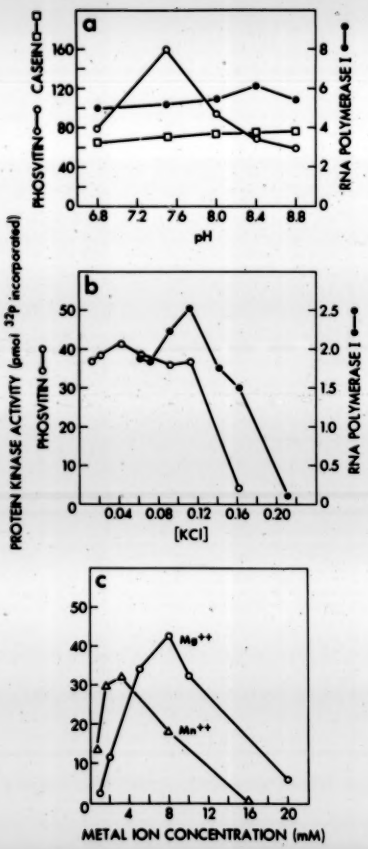
so could be composed of one or more of the four major polypeptides present in the DEAE-Sephadex pool. The specific activity of the protein kinase after DEAE-Sephadex chromatography is comparable to similar protein kinases purified by others (11,24) but is only 10% that reported for a yeast protein kinase purified by Lerch et al. (25). This latter enzyme exists as a monomer of 42,000 daltons, whereas the enzyme described here appears to be larger. The subcellular localization of this protein kinase remains to be determined but it has general enzymatic properties similar to those described by others for nuclear protein kinases (26-28). For example, it is not stimulated by cAMP, can use either ATP or GTP as a phosphate donor and is more active with acidic protein phosphate acceptors like phosvitin and casein than basic proteins like histones or protamine. Under standard assay conditions and an acceptor concentration of 4 mg/ml, initial velocities of 6.34, 3.11, 1.01 and 0.02 pmol of phosphate incorporated per minute were obtained respectively. Some of the other enzymatic properties of this kinase are summarized in Figure 4. The protein kinase has a broad pH optimum, is inhibited by KCl concentrations greater than 0.2 M and requires a divalent metal ion (Mg^{+2} or Mn^{+2}) for activity.

FIGURE 4. Enzymatic properties of yeast protein kinase. Enzyme activity was measured as described in the text. Each reaction contained 0.05 µg of protein kinase (DEAE-Sephadex A-25 pool) and either 0.20 mg of phosvitin or casein or 5.8 µg of yeast polymerase I.

a. Effect of pH on protein kinase activity. The pH of each reaction was adjusted with 0.05 M Tris-HCl. The KCl and $MgCl_2$ concentrations were 0.10 M and 5 mM respectively.

b. Effect of KCl on protein kinase activity. The pH of the reactions was 7.9. $MgCl_2$ concentration was 5 mM.

c. Effect of divalent metal ion concentration on protein kinase activity. The pH of the reactions was 7.9. KCl concentration was 0.10 M.


a
PHOSVITIN CASEIN
RNA POLYMERASE I
pH
b
PROTEIN KINASE ACTIVITY (pmol 32P incorporated)
PHOSVITIN
RNA POLYMERASE I
[KCl]
c
Mg++
Mn++
METAL ION CONCENTRATION (mM)

RESULTS

Simultaneous purification of RNA polymerases I, II and III from small quantities of cells, whether yeast or higher eucaryotes, has been difficult. We have been able to purify the three yeast RNA polymerases from 35 g of cells. A batchwise purification procedure (15) originally developed for the large scale purification of yeast polymerase I was adapted to accommodate small amounts of cells by reducing the amount of resin and buffer volumes used proportionately to the amount of yeast cells. The ion filtration column described in the original procedure was eliminated. Polymerase I was purified by batchwise absorption and elution from phosphocellulose and then DEAE-cellulose, and finally by high salt sucrose density gradient sedimentation. RNA polymerases II and III were not absorbed by phosphocellulose under the same conditions as polymerase I and were purified from the filtrate. We have utilized the synthetic polyanion, Polymin P, first used by Zillig (29) in the purification of E. coli RNA polymerase and subsequently by Burgess in the purification of E. coli RNA polymerase (30) and wheat germ polymerase II (31), to separate polymerases II and III from nucleic acid. The RNA polymerases II and III in the filtrate are first precipitated with the Polymin P with much of the DNA and RNA and then are selectively eluted. This step separates the polymerases II and III from the bulk of the nucleic acid and also affords an approximately eight fold purification with no loss of polymerase activity (22). The polymerases I and III were then separated by chromatography of the enzymes on DEAE-cellulose under conditions where only polymerase II is absorbed. The polymerase

II was eluted from the DEAE-cellulose and then purified by phosphocellulose chromatography and finally a high salt sucrose gradient. Polymerase III was purified from the DEAE-cellulose flow-through by chromatography on DEAE-Sephadex, then denatured DNA-cellulose and finally a high salt sucrose gradient. The final yield and purity of RNA polymerases I, II and III isolated by these procedures from small quantities of cells is comparable to those obtained with larger amounts. These purification procedures were then used to purify the polymerases from yeast grown in $^{32}P_i$.

RNA Polymerase I. The RNA polymerase I purified by this procedure is similar to the enzyme purified by other procedures (15,32). Figures 5 and 6 show the activity, protein concentration and polypeptide composition of fractions of the sucrose gradient, the final purification step. Sodium dodecyl sulfate-polyacrylamide gel analysis resolved the protein in the region of the polymerase activity (Fig. 6a, fractions 11-13) into 10 polypeptides (185,000, 137,000, 48,000, 44,000, 41,000, 36,000, 28,000, 24,000, 20,000 and 14,500 daltons (subunit 12,300 is not resolved under these conditions)) which have been identified by Valenzuela *et al.* (15) and Buhler *et al.* (32) as components of the enzyme. Thus the enzyme is essentially pure. When polymerase I was purified by this procedure from yeast cells grown in $^{32}P_i$, a significant fraction of the phosphate-labeled protein sedimented with the polymerase activity. This suggested that polymerase I contained phosphate (Fig. 6a). The relationship of the phosphate-labeled polypeptides to polymerase subunits was determined by autoradiography of the dried sodium dodecyl sulfate-polyacrylamide gel. The majority of the radioactivity migrated with

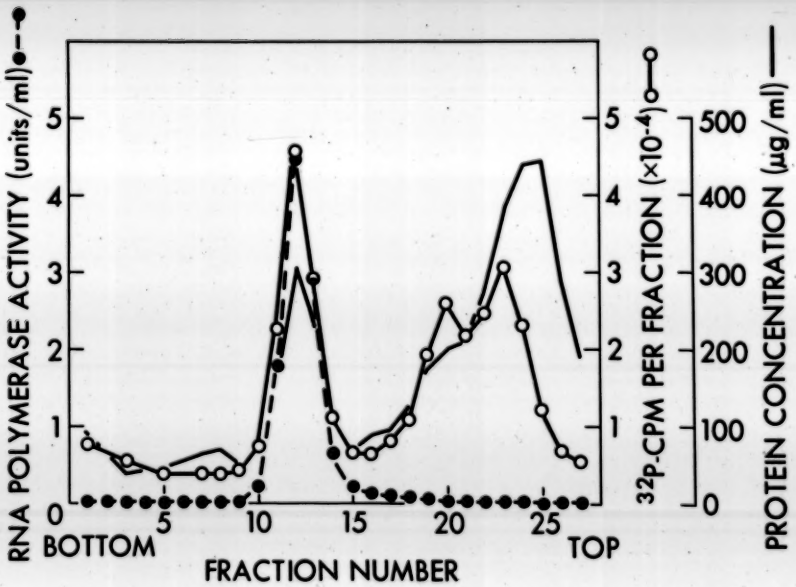
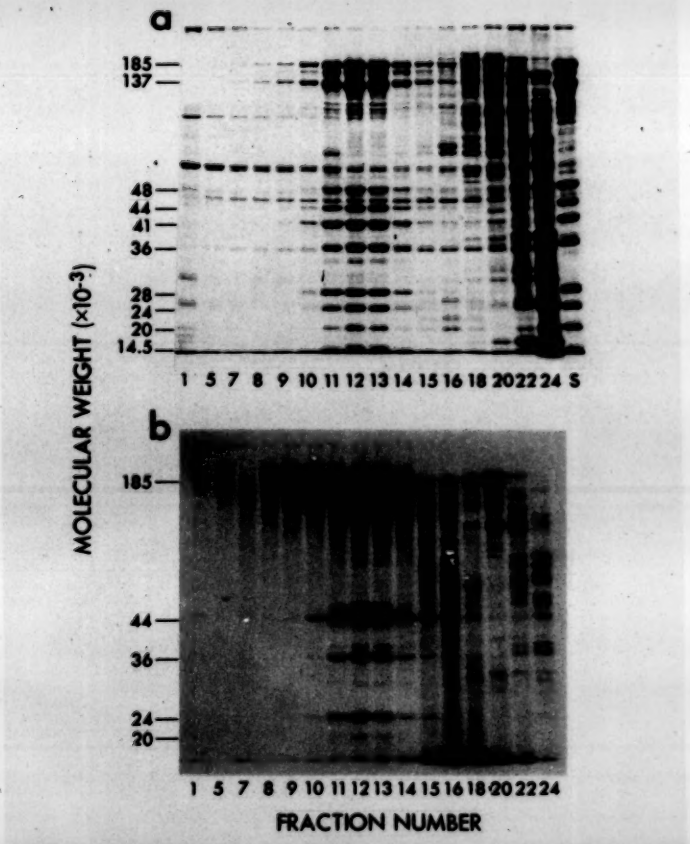


FIGURE 5. Sucrose density gradient centrifugation of phosphorylated yeast RNA polymerase I. The gradient was prepared, loaded and centrifuged as described in the text. Fractions were assayed for protein, RNA polymerase activity and ^{32}P-protein.

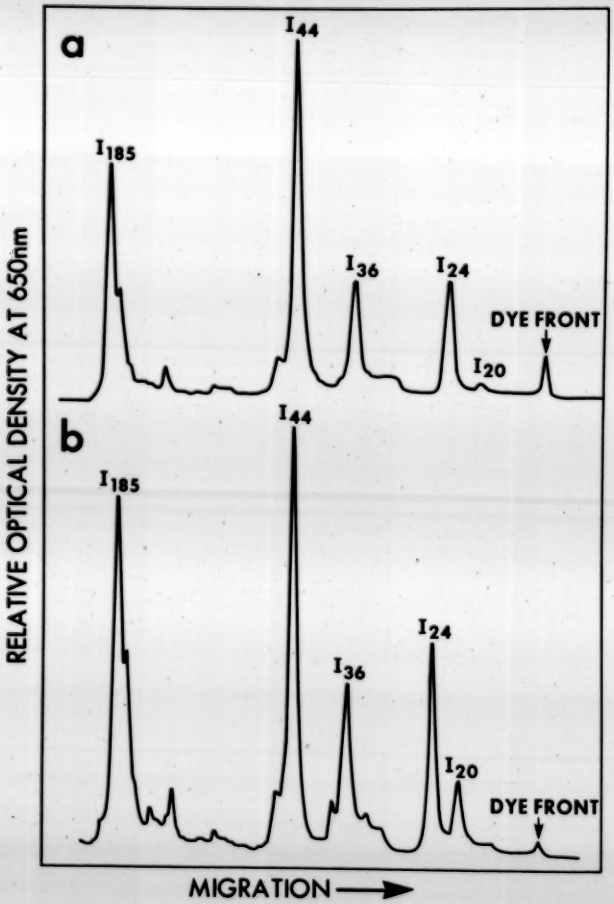
FIGURE 6. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of protein from sucrose gradient of yeast RNA polymerase I described in Figure 5. The enzyme subunits are designated by their molecular weights. The fraction numbers correspond to those of the gradient in Figure 5. The gel concentration was 10%. a. Stained polypeptides. The lane, designated S, is yeast RNA polymerase I reference. b. Autoradioradiogram of gel.


a
185
137
48
44
41
36
28
24
20
14.5
1 5 7 8 9 10 11 12 13 14 15 16 18 20 22 24 S
b
185
44
36
24
20
1 5 7 8 9 10 11 12 13 14 15 16 18 20 22 24
FRACTION NUMBER
MOLECULAR WEIGHT (×10⁻³)

five polymerase I polypeptides (185,000, 44,000, 36,000, 24,000 and 20,000 daltons (Fig. 6b and 7)). Besides these radioactive polypeptides, there is a small amount of radioactivity at the dye front. The majority of the label at the dye front probably represents contamination of the enzyme fractions with the material from the region of fractions 15-20.

Following partial acid hydrolysis of ^{32}P-polymerase I and analysis of the hydrolyzate by high voltage paper electrophoresis, approximately 7% of the radioactivity migrated with phosphothreonine, 22% with phosphoserine, 70% with inorganic phosphate and the remaining 1% remained at the origin. These data suggest that these amino acids are present in RNA polymerase I, but do not exclude the possibility that other amino acids might be phosphorylated. $^{32}P_i$ is produced under these hydrolysis conditions by decomposition of the phosphate ester of phosphoserine or phosphothreonine (21). Since the rate of decomposition of phosphoserine is approximately four times that of phosphothreonine, the fraction of the radioactivity in phosphoserine and, to a lesser extent, phosphothreonine is underestimated. N-phosphoamino acids also decompose under acidic conditions (33). The incorporated ^{32}P was acid stable (6% was released as $^{32}P_i$ in 15 min in 0.5 N HCl at 60°) and base labile (69% was released as $^{32}P_i$ in 15 min in 0.5 N NaOH at 60°) which suggests that most of the phosphate is attached to serine and threonine residues and not to lysine, arginine or histidine residues which are acid labile and base stable (33). Of the incorporated ^{32}P, 85% was sensitive to potato acid phosphatase and 65% was sensitive to bacterial alkaline phosphatase. It is possible that a small fraction of the phosphate label could be incorporated by adenylylation or ADP-ribosylation of enzyme polypeptides.

FIGURE 7. Densitometer tracing of autoradiograms of *in vivo* phosphorylated RNA polymerase I, purified as described in the text, obtained after resolution of polymerase polypeptides by electrophoresis in sodium dodecyl sulfate-10% polyacrylamide gels. a. The pattern of subunit phosphorylation observed when polymerase I was isolated from yeast cells grown in phosphate-depleted medium. b. The pattern of subunit phosphorylation observed when polymerase I was isolated from yeast cells grown in complete medium.


a
I_{44}
I_{185}
I_{36}
I_{24}
DYE FRONT
I_{20}
b
I_{44}
I_{185}
I_{24}
I_{36}
I_{20}
DYE FRONT
RELATIVE OPTICAL DENSITY AT 650nm
MIGRATION

We consider the latter unlikely since we have been unable to detect any NAD^+:protein ADP-ribosyltransferase activity in isolated yeast nuclei.

The 185,000 and 44,000 dalton polypeptides were more highly labeled than the others (Fig. 7). The 20,000 dalton polypeptide was only lightly labeled. The relative extent of phosphorylation of this subunit appears to be sensitive to the phosphate content of the culture medium since it is more highly labeled when the enzyme was isolated from cells grown in complete rather than phosphate-depleted medium (Fig. 7).

RNA Polymerase II. The RNA polymerase II purified as described in Experimental Procedures was 90-95% pure as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Figs. 8, 9a, fractions 13-15) and has the following polypeptide composition: 205,000, 145,000, 46,000, 33,500, 28,000, 24,000, 18,000, 14,500 and 12,500 daltons (the 12,500 subunit is not resolved in Fig. 9a but is evident in Fig. 12). The polypeptide composition of this enzyme preparation is similar to those described by ourselves (34) and others (32), except that the molecular weight of the largest subunit in these previous preparations was approximately 175,000 (Fig. 9a, column S). However, the apparent molecular weight of the largest subunit when polymerase II was isolated by this procedure and in a preparation recently described by Dezelee et al. (35) is similar to that of the large subunit of myosin, i.e., approximately 220,000 (36) (Fig. 9a, columns M and S and fraction 14). This subunit has an apparent molecular weight of 205,000 as determined by electrophoresis in a 6.5% sodium dodecyl sulfate-polyacrylamide gel which resolves high molecular weights polypeptides better than an 11% gel (data not shown). There is little of the 175,000 dalton polypep-

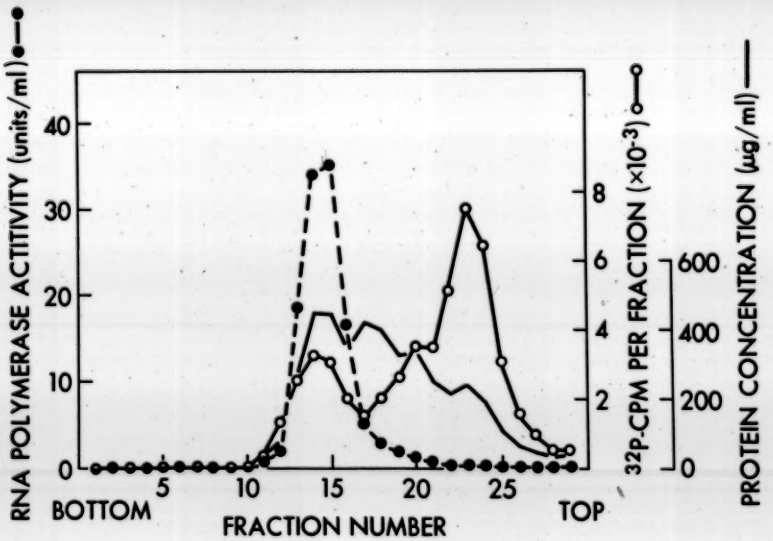
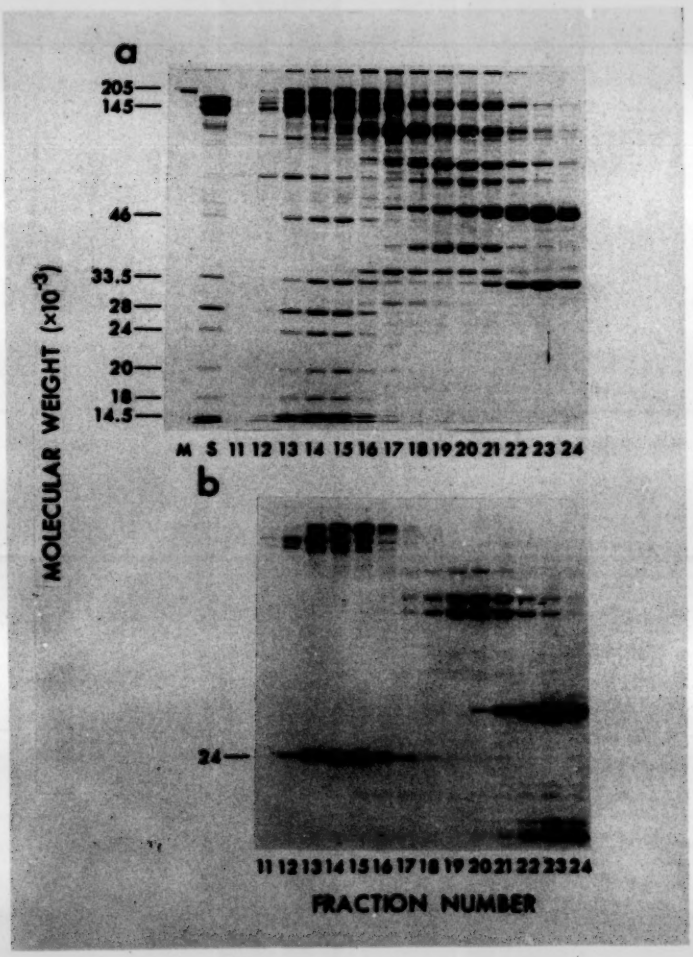


FIGURE 8. Sucrose density gradient centrifugation of phosphorylated yeast RNA polymerase II. The gradient was prepared, loaded and centrifuged as described in the text. Fractions were assayed for protein, RNA polymerase activity and ^{32}P-protein.

FIGURE 9. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of protein from sucrose gradient of yeast RNA polymerase II described in Figure 8. The enzyme subunits are designated by their molecular weights. The fraction numbers correspond to those of the gradient in Figure 8. The gel concentration was 11%. a. Stained polypeptides. The lane designated M is myosin, the lane designated S is yeast RNA polymerase II reference. b. Autoradiogram of gel.


a
205
145
46
33.5
28
24
20
18
14.5
MOLECULAR WEIGHT (×10⁻³)
M S 11 12 13 14 15 16 17 18 19 20 21 22 23 24
b
24
11 12 13 14 15 16 17 18 19 20 21 22 23 24
FRACTION NUMBER

tide evident in this preparation. This is consistent with an origin by proteolysis during isolation as proposed by Dezelee et al. (35). Thus, when proteolysis is efficiently prevented, the only form of polymerase II isolated is one possessing the 205,000 dalton subunit. There is no apparent difference in molecular weight in the other subunits in the polymerase II preparation (Fig. 9a, column S and fraction 14). These subunits do not appear to be as sensitive to proteolysis. Dezelee et al. (35) have found no significant differences in α-amanitin sensitivity or template preference between the two forms. The contaminants in this polymerase II preparation are several polypeptides with molecular weights between 46,000 and 145,000 that are relatively tightly associated with the polymerase since they sediment with the enzyme in 0.5 M KCl.

When polymerase II was purified by this procedure from yeast grown in $^{32}P_i$, analysis of the sucrose gradient showed a small peak of radioactivity sedimenting with the enzyme. The identity of the radioactive polypeptides was determined after sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the proteins in the gradient fractions by autoradiography. Most of the radioactivity which sedimented with polymerase migrated with the 24,000 dalton subunit (Fig. 9, fractions 14,15). In addition there were radioactive polypeptides with apparent molecular weights of 225,000, 165,000 and 140,000 present in minor amounts which do not migrate with any polymerase II subunits. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of ^{32}P-polymerase II in a 6.5% resolving gel gel and subsequent autoradiography also indicated that the three large phosphorylated polypeptides are not polymerase II subunits (data not shown). These large phosphorylated polypeptides do not sedi-

ment exactly with the enzyme which also suggests that they are contaminating polypeptides. Sucrose gradient centrifugation resolved polymerase II from a 33,500 dalton radioactive polypeptide. The enzyme subunit of this molecular weight is not labeled.

RNA Polymerase III. This enzyme has generally been difficult to purify; the procedure described here has been used to purify polymerase III from variable quantities of cells. After affinity chromatography on denatured DNA cellulose, polymerase III was approximately 80% pure (data not shown) and sucrose gradient sedimentation removed most of the remaining protein contaminants (Fig. 10 and 11a). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis resolved the protein in the active fractions into ten polypeptides (160,000, 128,000, 82,000, 41,000, 37,000, 34,000, 28,000, 24,000, 20,000 and 14,500 daltons) (Fig. 11a, fractions 14,15) which Valenzuela et al. (37) have previously designated as components of the enzyme.

When polymerase III was purified by this procedure from yeast grown in $^{32}P_i$, a large fraction of the radioactivity loaded on the sucrose gradient sedimented with the polymerase activity. The 24,000 and 20,000 dalton subunits of polymerase III were phosphorylated (Fig. 11, fractions 14,15). In addition, there were several phosphorylated polypeptides in the range of 47,000-53,000 that presumably are contaminants.

In Vitro Phosphorylation of Yeast RNA Polymerases. The purification and some properties of a yeast protein kinase which purifies with polymerase I are discussed in Experimental Procedures. This protein kinase, when incubated with purified RNA polymerases I, II and III and [γ-^{32}P]ATP, phosphorylates the polymerase subunits that are phosphorylated in vivo

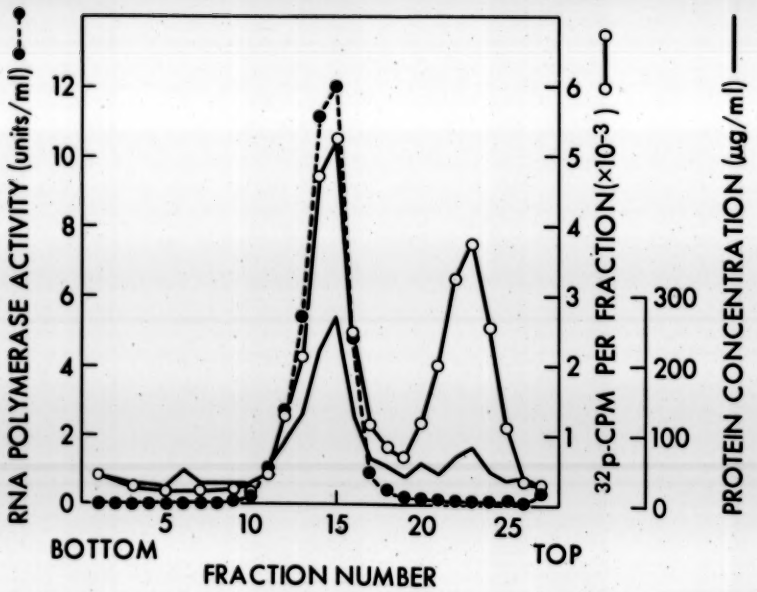
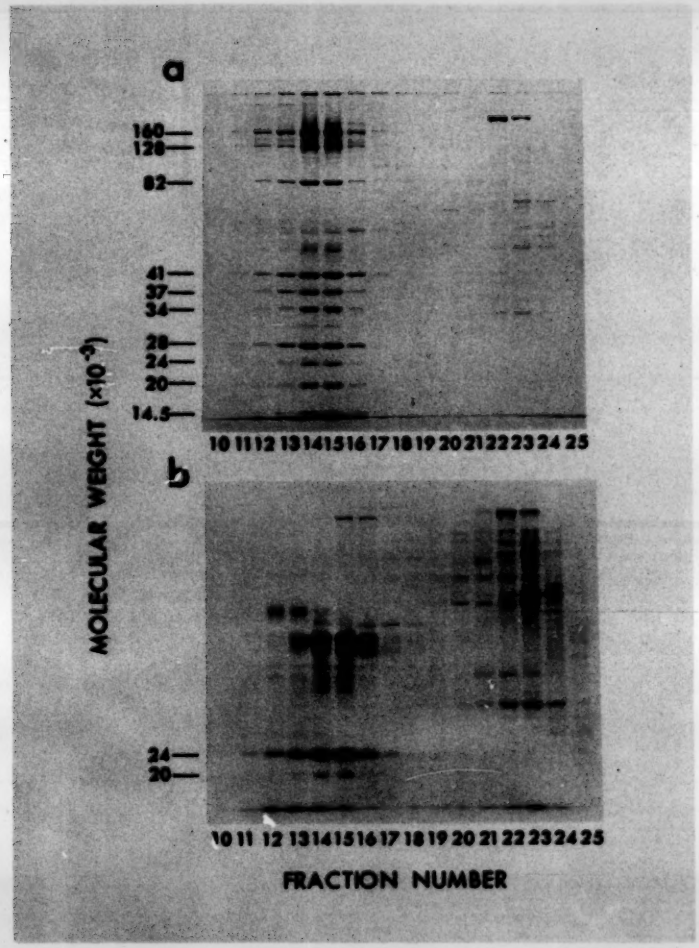


FIGURE 10. Sucrose density gradient centrifugation of phosphorylated yeast RNA polymerase III. The gradient was prepared, loaded and centrifuged as described in the text. Fractions were assayed for protein, RNA polymerase activity and ^{32}P-protein.

FIGURE 11. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of protein from sucrose gradient of yeast polymerase III described in Figure 10. The enzyme subunits are designated by their molecular weights. The fraction numbers correspond to those of the gradient in Figure 10. The gel concentration was 10%. a. Stained polypeptides. b. Autoradiogram of gel.


a
160
128
82
41
37
34
28
24
20
14.5
b
24
20
MOLECULAR WEIGHT (×10⁻³)
10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25
10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25
FRACTION NUMBER

($I_{185,000}$, $I_{44,000}$, $I_{36,000}$, $I_{24,000}$, $I_{20,000}$, $II_{24,000}$, $III_{24,000}$ and $III_{20,000}$) and also $I_{48,000}$ and $II_{33,500}$ daltons (Fig. 12). The protein kinase also phosphorylated contaminating polypeptides particularly in polymerase III. Since several groups have reported that protein kinases stimulate RNA polymerase I and II activity in a cell-free system presumably by phosphorylating the polymerase (9-11), we tested the effect of phosphorylation of polymerase on activity using purified yeast RNA polymerases I, II and III and protein kinase. Polymerase activity was measured with native yeast DNA as a template, conditions similar to those used by the other groups. As shown in Table II, the phosphorylation of purified polymerase I (0.4 moles of P were incorporated per mole of enzyme) resulted in a modest (14%) decrease in enzyme activity. Phosphorylation of yeast polymerase II (0.2-0.3 moles of P were incorporated per mole of enzyme) had no effect on enzyme activity as measured under these conditions with either $MgCl_2$ or $MnCl_2$. However, a heat-insensitive component of the protein kinase preparation caused a small inhibition of polymerase II activity. Phosphorylation of polymerase III (1.8 moles of P were incorporated per mole of enzyme) had no effect on enzyme activity under these conditions. The results presented in Table II suggest that phosphorylation of purified yeast RNA polymerase has little effect on activity. Since the enzymes are already phosphorylated when isolated, the effect of additional phosphorylation on activity might be reduced. We therefore tested the effect of acid and alkaline phosphatase on polymerase activity. There was no significant difference (%10%) in the activity of polymerase I in the presence of acid or alkaline phosphatase. Polymerase I, dephosphorylated by alkaline phosphatase, incorpor-

FIGURE 12. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of *in vitro* phosphorylated RNA polymerases I, II and III. Ten µg of each of the purified enzymes was phosphorylated with yeast protein kinase (0.125 µg, DEAE-Sephadex A-25 pool) under standard reaction conditions as described in the text except the reaction was allowed to proceed for 30 min. The reaction was terminated by the addition of an equal volume of 10% trichloroacetic acid. The precipitated protein was collected by centrifugation and subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis. The gel concentration was 11%. The enzyme subunits phosphorylated *in vitro* are designated by their molecular weights. Lanes 1 and 5 are of polymerase I, 2 and 6 are of polymerase II, 3 and 7 are of polymerase III, and 4 and 8 are of the protein kinase alone.

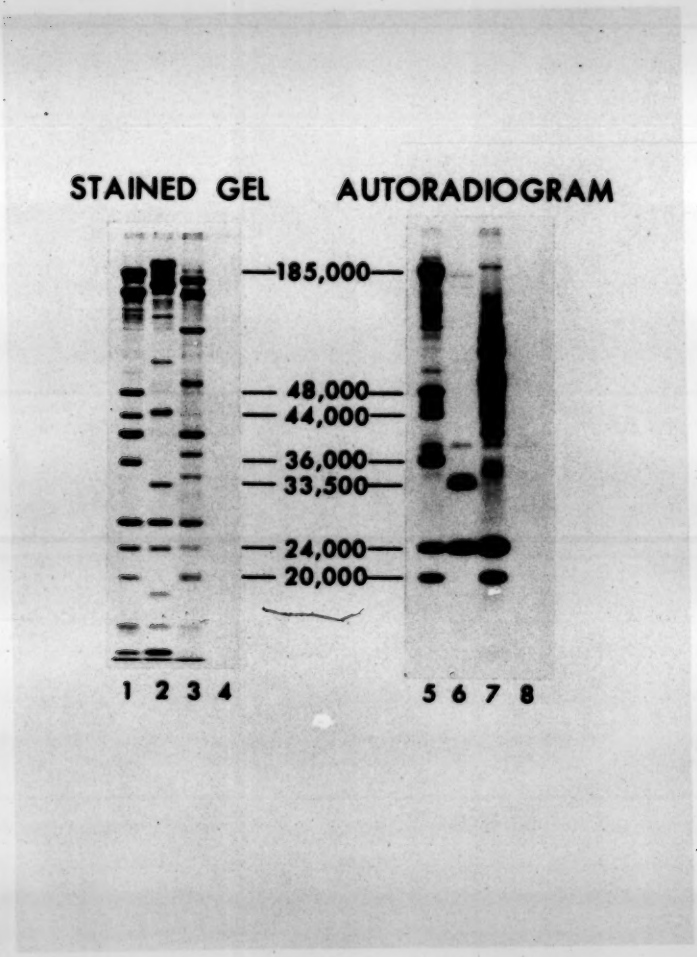

STAINED GEL
AUTORADIOGRAM
185,000
48,000
44,000
36,000
33,500
24,000
20,000
1 2 3 4
5 6 7 8

Table II. Purified RNA polymerases I, II and III, 1.16 µg (2 pmol), 2.25 µg (4.5 pmol), and 1.52 µg (2.5 pmol), respectively, were incubated with yeast protein kinase (0.05 µg, DEAE-Sephadex A-25 pool), heat-inactivated (5 min, 100°) protein kinase (0.05 µg, DEAE-Sephadex A-25 pool) or without additions in an incubation mixture of 0.050 ml which contained 50 mM Tris-HCl, pH 7.9, either 10 mM $MgCl_2$ or 3.2 mM $MnCl_2$, 0.01 mM [γ-^{32}P]ATP(1475 cpm/pmol), 10 mM 2-mercaptoethanol, 0.1 M KCl and 10% glycerol. The phosphorylation was allowed to proceed for 15 min at 30°, and then the polymerase activity was assayed. The final incubation mixture contained in 0.10 ml: 60 mM Tris-HCl, pH 7.9, either 5 mM $MgCl_2$ or 1.6 mM $MnCl_2$, 0.60 mM each of [γ-^{32}P]ATP (25 cpm/pmol), GTP and CTP, 0.01 mM [^{3}H]UTP (675 cpm/pmol), 10 mM 2-mercaptoethanol, 0.1 M KCl, 9.4 µg of native yeast DNA and 5% glycerol. After incubation for 10 min at 30°, 0.090 ml aliquots were applied to Whatman 3 MM filter discs which were washed as described for the protein kinase assay and counted as described elsewhere (15). In these reactions, the incorporation of ^{32}P into acid insoluble material reflects the amount of polymerase phosphorylation whereas the incorporation of [^{3}H]UMP into acid insoluble material indicates the amount of RNA synthesis. There was no incorporation of ^{32}P into acid insoluble material in the presence of protein kinase alone under these conditions.

TABLE II

Effect of *In Vitro* Phosphorylation of Purified RNA Polymerases on Activity

Enzyme	Divalent metal ion	Addition	^{32}P-Phosphate incorporated (pmol)	^{3}H-UMP incorporated (pmol)	% Control polymerase activity	Moles P incorporated per mole of polymerase
Pol I	$MgCl_2$	None	0	5.41	98	
		Heat-inactivated protein kinase	0	5.52	100	
		Protein kinase	0.80	4.86	86	0.40
Pol II	$MgCl_2$	None	0	1.13	116	
		Heat inactivated protein kinase	0	0.98	100	
		Protein kinase	1.30	0.98	100	0.29
Pol II	$MnCl_2$	None	0.04	17.46	105	
		Heat inactivated protein kinase	0.02	16.66	100	
		Protein kinase	1.00	16.68	100	0.22
Pol III	$MgCl_2$	None	0.05	15.38	102	
		Heat inactivated protein kinase	0.03	15.09	100	
		Protein kinase	4.42	15.27	101	1.77

ated three times as much phosphate on a molar basis as the control enzyme. Thus the ability to accept phosphate varies, as expected, with the degree of phosphorylation.

DISCUSSION

Phosphorylation of chromosomal proteins has been suggested as one mechanism for the regulation of gene expression in eucaryotes. This hypothesis is based on the fact that there is a correlation between phosphorylation of certain nonhistone chromosomal proteins and gene activation (38). The observation that bacterial RNA polymerase is phosphorylated upon bacteriophage infection (39), as well as reports which suggested that eucaryotic RNA polymerases might be phosphorylated in cell-free systems (9-11), caused us to examine whether phosphorylation regulates RNA polymerase activity in yeast, which is a more experimentally tractable system. We have shown that all three polymerases are phosphorylated, have identified the phosphorylated subunits, and have isolated a protein kinase which can catalyze this modification.

In order to determine if yeast RNA polymerases are phosphorylated *in vivo*, it was necessary to develop procedures for the purification of these enzymes from small quantities of cells. Procedures were developed for the simultaneous purification of the three enzymes. These procedures are highly reproducible, can be scaled to any limits and we now believe them to be the method of choice for the isolation of the three RNA polymerases from yeast. They may be of general utility in the isolation of these enzymes from other eucaryotic sources as well.

The three enzymes were isolated from yeast cells grown continuously in $^{32}P_i$. The purified RNA polymerases I, II and III contained ^{32}P. Chemical analysis of ^{32}P-polymerase I indicated that the incorporation of P by the enzymes is primarily due to the phosphorylation of serine and, to a lesser extent, threonine residues. The phosphorylation of *E.*

coli RNA polymerase on T7 bacteriophage infection occurs primarily on threonine residues (39). The phosphorylated enzyme subunits were identified according to the following criteria. First, the phosphorylated polypeptide must sediment with the purified RNA polymerase in the sucrose gradient step. Second, the phosphorylated polypeptide must migrate with the stained polymerase subunit in a sodium dodecyl sulfate polyacrylamide gel and third, it should be possible to demonstrate in vitro phosphorylation of the subunits phosphorylated in vivo by an enzyme present in the cell extract. It is necessary to use both criteria one and two since our data indicate that the purified enzymes are contaminated with phosphorylated polypeptides which sediment with the enzyme activity in the sucrose gradient but do not migrate with enzyme subunits in a polyacrylamide gel. These polypeptides may be minor contaminants but can be very highly labeled. Criterion three is insufficient of itself to establish in vivo phosphorylation of the enzymes since protein kinases can phosphorylate subunits in vitro which are not phosphorylated in vivo. Using these criteria, the following polymerase subunits were phosphorylated in vivo: $I_{185,000}$, $I_{44,000}$, $I_{36,000}$, $I_{24,000}$, $I_{20,000}$, $II_{24,000}$, $III_{24,000}$, $III_{20,000}$.

The stoichiometry of the in vivo phosphorylation of the RNA polymerase is difficult to determine precisely but it is possible to obtain approximate values. The enzymes were purified from yeast cells grown in inorganic phosphate depleted medium containing $^{32}P_i$. Under these conditions, the inorganic phosphate content of the medium is no higher than 10^{-4} M (13). Our determinations suggest that it may be approximately 2×10^{-5} M. Using these values for the inorganic phosphate concentration and neglecting the contribution of the organic phosphate in

the medium which enters the pool of inorganic phosphate after hydrolysis by an inducible phosphomonoesterase (14), the stoichiometry of the phosphorylation reaction can be estimated from the amount of ^{32}P incorporated into the purified enzymes. These estimations indicate that there are 2.5-12 moles of P incorporated per mole of polymerase I, 0.2-1.0 moles of P per mole of polymerase II, and 0.4-2.0 moles of P per mole of polymerase III. This range of values are compatible with the thesis that all enzyme molecules are phosphorylated. Also, the relative disparity in the labeling of individual subunits (especially polymerase I (Fig. 7)) suggests that some subunits may be phosphorylated at more than one site.

A consideration of the function of the phosphorylated polymerase subunits is relevant to the physiological role of phosphorylation. Polymerase I is more extensively phosphorylated than the other two enzymes suggesting that phosphorylation may be involved in the control of ribosomal RNA precursor synthesis. Moreover, the phosphorylated subunits present in polymerases II and III are not unique to these enzymes but are also present in polymerase I. In particular, the 24,000 dalton subunit of each enzyme is phosphorylated. This subunit by peptide mapping and two-dimensional gel electrophoretic analysis appears to be identical (32,40) in all enzymes. Both biochemical (41) and genetic evidence (42) suggests that it may be necessary for catalytic activity. The phosphorylated subunits $I_{20,000}$ and $III_{20,000}$ also appear to be identical polypeptides (40). Subunits $I_{44,000}$ and $I_{36,000}$ dissociate readily from polymerase I and may be necessary (possibly in conjunction with subunit $I_{48,000}$) for activity on native DNA templates (15,34,43,44). Subunit $I_{185,000}$ was the only large subunit of the three enzymes that

was phosphorylated. The role of the large subunits in the yeast enzymes has not been established but in bacterial RNA polymerases they contain the active center for the polymerization reaction (45). It is evident that phosphorylation of subunits could be one way of controlling transcriptive activity; however, the physiological role of this modification is not established.

We have been unable to demonstrate a significant effect of phosphorylation on RNA polymerase activity using conventional assay procedures. Other groups have reported that protein kinases stimulate the activity of partially purified RNA polymerases I and II and have proposed positive regulation by this means (9-11,46). However, none of these previous studies have demonstrated phosphorylation of the enzyme protein according to the criteria used in this study. Their conclusions are therefore subject to question. For example, the observed stimulation of polymerase activity may not be due to phosphorylation of the enzyme itself but to the phosphorylation of contaminating proteins (47,48).

We believe it is necessary to develop more incisive experimental means to test the physiological role of phosphorylation. Since RNA polymerase is a complex molecule which interacts with DNA, RNA and presumably other chromosomal proteins *in vivo*, phosphorylation of serine and threonine residues which introduces negative charge onto the molecule could alter both inter- and intramolecular interactions. Therefore it will be necessary to measure parameters of polymerase function besides activity on DNA: for example, affinity for DNA, correct initiation and termination of transcription of specific genes using DNA and chromatin templates, and enzyme stability. Future experiments will be designed to measure these more specific aspects of polymerase function. *In vivo*

phosphorylation experiments should also be continued in order to develop physiological correlates between patterns of polymerase phosphorylation and alterations in the synthesis of specific classes of RNA and so help to establish the role of phosphorylation in the regulation of transcription.

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Yeast DNA-dependent RNA Polymerase I

A RAPID PROCEDURE FOR THE LARGE SCALE PURIFICATION OF HOMOGENEOUS ENZYME*

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A procedure has been developed for the rapid purification of large amounts of yeast RNA polymerase I (A). The method involves batchwise treatment with phosphocellulose and DEAE-cellulose, ion filtration chromatography on DEAE-Sephadex, sucrose gradient centrifugation, and DNA-cellulose chromatography. The enzyme obtained is apparently homogeneous by sedimentation velocity analysis and has a specific activity of 300 nmol of UMP incorporated into RNA in 10 min per mg of protein. Between 30 and 45 mg of enzyme can be obtained in 5 days from 3.0 kg of yeast cells.

The subunit composition of the enzyme was determined by polyacrylamide gel electrophoresis in the presence of 0.1% sodium dodecyl sulfate. The purified polymerase is composed of 11 putative subunits with molecular weights 185,000 (I_a), 137,000 (I_b), 48,000 (I_c), 44,000 (I_d), 41,000 (I_e), 36,000 (I_f), 28,000 (I_g), 24,000 (I_h), 20,000 (I_i), 14,500 (I_j), and 12,000 (I_k).

Yeast polymerase I separates into two forms when subjected to gel electrophoresis under nondenaturing conditions. The main component which migrates faster contains all the subunits except the polypeptides I_c and I_f. The slow migrating component which is present in lower amounts contains all the subunits.

Since the original reports of the existence of multiple forms of RNA polymerases (I, II, and III) in sea urchin, rat liver, and in yeast (1–4), there have been confirmatory findings in virtually all other eukaryotic cells investigated. The purification and subunit structure of some of the enzymes have also been reported (5–8).

Large quantities of homogeneous protein are required for establishing the structure-function relationship of the complex RNA polymerase molecule and for the *in vitro* study of the mechanism and specificity of transcription in eukaryotic systems.

Yeast is one of the most suitable sources for large scale purification of eukaryotic RNA polymerases since it is readily available at reasonable cost in bulk amounts, growth conditions can easily be manipulated, and yeast genetics is well developed and many well characterized mutants are available (9, 10).

A number of procedures have been reported for the purification of yeast polymerases I and II (11–15), but none is satisfactory, for the methods employed are not easily scaled up, the yields are low, or the specific activity of the product is low. Finally, there may be contamination with polymerase III.

We report here a method for the facile preparation of large quantities of polymerase I in high yield. We have utilized batchwise absorption on phosphocellulose and DEAE-cellulose modified from the procedure of Buhler *et al.* (15), followed by ion filtration chromatography on DEAE-Sephadex (16), and sucrose gradient centrifugation in 25% glycerol. DNA-cellulose chromatography has been added as a final step to remove contaminating polymerase III and to yield enzyme homogeneous by native gel electrophoresis and sedimentation velocity. Dialysis has been eliminated and dilution of the enzyme is avoided. Smaller quantities of highly purified RNA polymerase III can also be recovered as a by-product. We also report measurements of the size and stoichiometry of the polypeptides present in the RNA polymerase I which reveal a complex quaternary structure.

EXPERIMENTAL PROCEDURES

Materials—[^{3}H]UTP (20 Ci/mmol) was obtained from New England Nuclear, Boston, Mass. Dimethylsulfoxide, calf thymus DNA, and phenylmethylsulfonylfluoride were purchased from Sigma Chemical Co., St. Louis, Mo. Dithiothreitol, pyruvate kinase, and phosphoenolpyruvic acid were from Calbiochem, La Jolla, Ca. Nucleotides were from P-L Biochemicals, Milwaukee, Wis. Phosphocellulose (Whatman P_{11}), 7.4 meq/g, and DEAE-cellulose (Whatman DE52), 1.0 meq/g, were purchased from Reeve Angel, New York, N. Y. Sucrose, bidistilled glycerol, and ammonium sulfate were enzyme grade reagents. α-Amanitin was a Boehringer product obtained from Henley Co., New York, N. Y. *Saccharomyces cerevisiae* yeast cells were the kind gift of Red Star Yeast Co., Oakland, Ca.

Preparation of Resins—Phosphocellulose and DEAE-cellulose were precycled and equilibrated as described by Buhler *et al.* (15). Denatured calf thymus DNA-cellulose was prepared by the method of

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Alberts and Herrick (17). After extensive washing the resin contained 0.3 mg of DNA/ml of resin.

Salt, DNA, and Protein Measurements.—Conductivity was measured with a Radiometer CDM2 conductivity meter following a 100-fold dilution in water, and salt concentration determined by reference to a standard curve. DNA was measured by the method of Burton (18). Protein was determined after precipitation with 10% trichloroacetic acid by the method of Lowry *et al.* (19) using crystalline bovine serum albumin (Pentex type I) as standard.

RNA Polymerase Assay—The standard incubation mixture contained in 0.060 ml: 60 mM Tris-HCl, pH 7.9, 1.6 mM $MnCl_2$ or 10 mM $MgCl_2$, 0.6 mM ATP, CTP, and GTP, 0.01 mM UTP, 0.5 μCi of [^{3}H]-UTP, 10 mM NaF, 10 mM 2-mercaptoethanol, 120 μg of bovine serum albumin, 0.1 mM EDTA, 10% glycerol, and 8 μg of native calf thymus DNA. For assay of RNA polymerase in crude extracts the incubation mixture included 0.2 μg of crystalline pyruvate kinase and 70 mM phosphoenolpyruvic acid. This reaction mixture containing nonsaturating levels of UTP was used in routine assays. However, when the specific activities of the different fractions were measured, the UTP concentration was raised to 0.6 mM. After incubation for 10 min at 30°, a 50-μl aliquot was withdrawn from each tube and applied directly onto Whatman DE81 filter discs. The filters were washed seven times with 5% Na_2HPO_4, twice with water, twice with ethanol, and dried. Radioactivity was measured by immersing the discs in 3 ml of a solution of 4 g/liter of Omnifluor (New England Nuclear) in toluene. One unit of activity corresponds to the incorporation of 1 nmol of UMP into RNA/10 min under the above conditions. The specific activity of UTP in the assay mixture was determined on an aliquot of the reaction mixture (without enzyme) which was spotted on a disc, dried, and counted. Due to the higher counting efficiency of [^{3}H]UMP in RNA than [^{3}H]UTP alone, samples were subjected to hydrolysis and solubilization by overnight treatment with 250 μl of a 1:6 mixture of water and NCS (Nuclear Chicago Solubilizer) and the resultant solutions counted in toluene fluor.

Polyacrylamide Gel Electrophoresis—The 9% and 12% acrylamide gel electrophoresis in 0.1% sodium dodecyl sulfate was carried out in slab gels (12 × 15 cm × 1.5 mm) (Hoefer Scientific Instruments, San Francisco, Ca.). Buffers and solutions were prepared according to Laemmli (20). Samples were ordinarily diluted 1:1 with 2 times concentrated sample buffer (20% glycerol, 10%, v/v, 2-mercaptoethanol, 6%, w/v, sodium dodecyl sulfate, and 0.125 M Tris-HCl buffer, pH 6.8). When too dilute, the samples were mixed with 20 μg of polyadenylic acid, precipitated with 10% trichloroacetic acid, and centrifuged 20 min at 2,000 × *g*. After removing the supernatant by aspiration the precipitate was washed twice with 1/1 ethanol/ether, once with ether, dissolved in 25 to 40 μl of sample buffer, and heated 3 min at 100°. The samples were layered over a stacking gel (3% acrylamide, 0.08% bisacrylamide, and 0.1% (v/v) tetramethylethylenediamine) and subjected to electrophoresis at 75 volts. When the sample entered the resolving gel (9% or 15% acrylamide, 0.27% or 0.4% bisacrylamide, and 0.05% (v/v) tetramethylethylenediamine) the voltage was increased to 110 volts. Gels were fixed by shaking them in 50% isopropyl alcohol/10% trichloroacetic acid for 30 min at 23° and stained 12 hours in 25% isopropyl alcohol-10% trichloroacetic acid-0.1% Coomassie blue at 23°. Gels were destained at 23° with 10% acetic acid. The slab was cut into strips (0.8 × 10 cm) and scanned at 550 or 650 nm with a Gilford linear transport device attached to a Gilford spectrophotometer. The relative amount of each component present was determined from the areas under the peaks. To calibrate the relationship between mobility and molecular weight for this system, a number of proteins of known molecular weights were subjected to electrophoresis under the same conditions. The following proteins and molecular weight values were used: *Escherichia coli* RNA polymerase subunit β′, 165,000; *E. coli* RNA polymerase β, 155,000; β-galactosidase, 130,000; phosphorylase A, 95,000; *E. coli* RNA polymerase σ, 85,000; bovine serum albumin, 65,000; *E. coli* RNA polymerase α, 40,000; carbonic anhydrase, 30,000; and lysozyme, 14,400.

Electrophoresis under nondenaturing conditions was carried out in cylindrical or slab acrylamide gels prepared as described by Maizel (21). In tubes electrophoresis was done at 0.3 mA/gel through the stacking portion (3% acrylamide) and at 2mA/gel through the resolving portion (5% acrylamide). When slabs were used (1.5-mm thick), stacking was done at 70 volts and separation at 200 volts. Gels were stained by stirring them 12 hours with 0.2% Coomassie blue in 5/1/5 (v/v) ethanol/acetic acid/water. For destaining the gels were stirred in the same solution without dye at 23°. Gel electrophoresis in two dimensions was carried out in slab gels. Native enzyme was used in the first dimension in 5% acrylamide gels under nondenaturing conditions (21). Sodium dodecyl sulfate gel electrophoresis was performed in the second dimension. The corresponding strip was cut, equilibrated 30 min at 23°, and 5 min at 80° in Laemmli's sample buffer without glycerol (20), and layered on top of a polymerized 3% acrylamide/0.1% sodium dodecyl sulfate stacking gel and 9% or 12% acrylamide/0.1% sodium dodecyl-sulfate resolving gels.

Assay of DNase and RNase Activities—DNase activity was monitored using the SV40 DNA allomorph conversion assay. RNA polymerase was incubated 10 min at 30° with SV40-[^{32}P]DNA (gift of Dr. H. Goodman) forms I and II in the normal polymerase assay mixture lacking [^{3}H]UTP and DNA. After stopping the reaction with 0.1% sodium dodecyl sulfate, the intactness of the DNA was analyzed by centrifugation (2.5 hours, 60,000 rpm, 10°) in a 5 to 20% sucrose gradient. Fractions from the gradient were precipitated with 10% trichloroacetic acid in the presence of bovine serum albumin, collected in glass-fiber discs, dried, and counted.

RNase activity was assayed by measuring the counts liberated after incubation of the enzyme under assay conditions, with ribosomal [^{3}H]RNA linked to small cellulose discs. [^{3}H]RNA was prepared by phenol extraction of polysomes from rat hepatoma cells previously labeled with [^{3}H]uridine. [^{3}H]RNA was covalently attached to the discs using a water-soluble carbodiimide as described by Gilham (22).

Sedimentation Velocity—Analytical sedimentation velocity studies were performed in a Beckman model E ultracentrifuge equipped with a photoelectric scanner. Sedimentation was followed with absorption optics at 280 nm using double sector cells. Samples were equilibrated with 0.05 M Tris-HCl, pH 7.9, 0.2 M KCl, 0.01 M $MgCl_2$, and 0.007 M 2-mercaptoethanol by passage through a column of Sephadex G-75. Sedimentation coefficients were calculated from the observed S values by correcting for the viscosity and density of the solutions (23). Density of solutions was determined by weighing volumes in a H. E. Pederson micropipette, calibrated with water at the same temperature.

RESULTS

Enzyme Purification

A summary of the purification procedure is presented in Table I. A detailed description of each step in the procedure follows.

Preparation of Cell Extract—Yeast cells in late log phase were harvested by low speed centrifugation, washed by resuspending first in cold distilled water, and then in 2 liters of extraction buffer (0.02 M Tris-HCl, pH 8.0, 10% glycerol, 0.01 M $MgCl_2$, 0.01 M 2-mercaptoethanol, 0.5 mM EDTA, 0.3 M ammonium sulfate, 1 mM phenylmethylsulfonyl fluoride, and 1% dimethylsulfoxide) per 1000 g, wet weight, of cells. The washed cells are suspended in a small amount of extraction buffer and the heavy paste is added dropwise to liquid N_2 and the pellets stored at −80°. Cells prepared in this way can be stored for several months with no noticeable change in RNA polymerase activity. The yeast extract was prepared by the method of Bhargava and Halvorson (24) as modified by Hager and Holland of this laboratory.[1] The cells are disrupted by crushing the frozen pellets through a 200-ml Eaton pressure cell (25) at a pressure of 10,000 psi and cooled to −30°. The broken cells are stirred with 2,200 ml of extraction buffer and the pH is adjusted to 8.0 with solid Tris. Unless otherwise stated all RNA polymerase purification steps were performed at 0–4°. The homogenate is centrifuged 60 min at 27,000 × *g* (Sorvall rotor GSA). The supernatant is removed and centrifuged for 2 hours at 50,000 × *g* in Beckman ultracentrifuge (rotor 21). The clear part of the supernatant is pooled to give about 2.5 liters of Fraction 1. On the average this fraction contains 30 mg/ml of protein. At this stage, due to the high amounts of RNA and presence of hydrolytic enzymes, the RNA polymerase assay is not reliable and gives about 20,000 total

[1] G. L. Hager and M. Holland, unpublished results.

TABLE I

Summary of purification of yeast RNA polymerase I

The data reported in this table were taken from one representative experiment. Of wet weight frozen yeast cells, 1000 g were used as starting material. One unit corresponds to 1 nmol of UMP incorporated in 10 min at 30°.

Purification step	Volume	Total protein	Activity	Specific activity	Yield
	ml	*mg*	*units*	*units/mg*	*%*
Extract	2,440	73,600	20,990[a]	0.28	
Phosphocellulose batch	1,300	650	21,200[a]	32	100
DEAE-cellulose batch	900	116	12,380[a]	107	58.4
Ion filtration (DEAE-Sephadex)	53	35	6,840[a]	195	32.3
Sucrose gradient	19.5	14.6	4,380	300	20.7
DNA-cellulose	25	10	3,000	300	14.1[b]

[a] RNA polymerases I and III are not separated at these stages. After ion filtration and sucrose gradient the amount of enzyme III is approximately 10% and 5%, respectively, of total polymerase units.

[b] The final yield (14.1%) is calculated on the basis of α-amanitin-insensitive activity (polymerase I and III activity). Polymerase III activity in the extract may be about 50% (G. L. Hager and M. Holland, unpublished results) so that the final yield may be as much as 28%.

α-amanitin-resistant units/1,000 g of cells. In recent preparations this high speed centrifugation step has been eliminated without compromising the yield and specific activity of the final product. The low speed supernatant is treated directly with phosphocellulose as described below.

Treatment with Phosphocellulose—Fraction 1 (2.5 liters) was added to 500 g (wet weight) of phosphocellulose previously equilibrated in Buffer A containing 0.15 M ammonium sulfate (Buffer A is 0.02 M Tris-HCl, pH 8.0, 0.01 M 2-mercaptoethanol, 0.5 mM EDTA, and 10% glycerol). After thorough mixing the ammonium sulfate concentration is reduced to 0.15 M by addition of 1 volume of Buffer A (2.5 liters). The suspension is slowly stirred for 30 min and then filtered through filter paper in a Buchner funnel without allowing the phosphocellulose to dry. The cake is suspended in 4 liters of Buffer A containing 0.15 M ammonium sulfate, stirred for 10 min, and collected by filtration. This procedure is repeated three more times. After the last filtration the phosphocellulose cake is suspended in 1.2 liters of Buffer A containing 0.4 M ammonium sulfate, stirred slowly for 30 min, and filtered as above. About 1.3 liters of filtrate are obtained, which contain RNA polymerase with a specific activity of 32 units/mg of protein (Fraction 2).

Treatment with DEAE-cellulose—Of DEAE-cellulose previously equilibrated in Buffer B containing 0.1 M ammonium sulfate (Buffer B is 0.02 M Tris-HCl, pH 8.4, 0.01 M 2-mercaptoethanol, and 0.5 mM EDTA), 400 g (wet weight) are added to Fraction 2 (1.3 liters). After thorough mixing the ammonium sulfate concentration is lowered to 0.1 M by addition of 3 volumes of Buffer B. The suspension is gently stirred for 30 min, filtered as above, and washed four times with 2 liters of Buffer B containing 0.1 M ammonium sulfate. RNA polymerase is eluted by suspending and stirring the cellulose cake in 800 ml of Buffer B containing 0.3 M ammonium sulfate for 30 min. After filtration 900 ml of enzyme solution with a specific activity of 106 units/mg of protein is obtained (Fraction 3). At this stage the enzyme can be conveniently stored by precipitating it with ammonium sulfate (see below) and dissolving in Buffer B containing 50% glycerol.

Ion Filtration Chromatography—RNA polymerase (Fraction 3) is precipitated by addition of 35 g of solid ammonium sulfate/100 ml of solution. After dissolution of the salt the precipitate is left overnight at 4° and collected by centrifugation (27,000 rpm for 45 min in rotor SW-27 of Beckman ultracentrifuge). The pellet is dissolved in 12 ml of Buffer B containing 30% glycerol and 0.35 M ammonium sulfate and loaded onto a column (4 × 30 cm) packed with DEAE-Sephadex A-25. If the preparation is started with 2,000 g of cells, the pellet is dissolved in 20 ml of buffer and a column (4 × 50 cm) is used. The column was previously equilibrated with 0.02 M Tris-HCl, pH 8.0, 25% glycerol, 0.5 mM EDTA, 0.02 M 2-mercaptoethanol, and 0.1 M ammonium sulfate. The column is developed with the same buffer containing 0.35 M ammonium sulfate (Fig. 1). The fractions containing activity are pooled (53 ml). The enzyme obtained has a specific activity of 195 units/mg of protein (Fraction 4). The experimental conditions for purifying RNA polymerases by ion filtration chromatography were developed by Michael Goldberg in this laboratory.

Sucrose Gradient Centrifugation—Fraction 4 is precipitated by overnight dialysis against Buffer B saturated with ammonium sulfate. The precipitate is collected by centrifugation (Beckman SW-27 rotor, 27,000 rpm, 45 min) and dissolved in a final volume of 3.6 ml with Buffer B containing 20% glycerol and 0.2 M KCl. Aliquots of 0.6 to 1.0 ml are layered on 11 ml of 5 to 20% (w/v) sucrose gradients in 0.05 M Tris-HCl, pH 8.0, 25% glycerol, 0.5 mM EDTA, 0.2 M KCl, and 0.02 M 2-mercaptoethanol. The gradients are centrifuged at 40,000 rpm for 28 hours at 4° (in a Spinco SW-41 rotor). A profile of the sucrose density is shown in Fig. 2. The enzyme pooled from the active fractions has a specific activity of 300 units/mg and a protein concentration of 0.75 to 1.5 mg/ml. It is stored at −80° (Fraction 5).

The conditions of the sucrose gradient centrifugation step were obtained from a study in which the protein load, nature and concentration of salt, and the glycerol concentration were systematically varied. The best yield and reproducibility was obtained with gradients containing protein loads of at least 6 mg/tube, 0.2 to 0.3 M KCl, and no Mg^{2+}. At salt concentrations lower than 0.2 M KCl partial aggregation of the enzyme occurs resulting in broadening of the protein peak.

DNA-cellulose Chromatography—Fraction 5 is diluted three times with Buffer C (0.02 M Tris-HCl, pH 8.0, 25% glycerol, 0.1 mM EDTA, and 0.02 M 2-mercaptoethanol) to bring the KCl concentration to 0.066 M and applied to a 25-ml column of denatured DNA-cellulose previously equilibrated with Buffer C containing 0.066 M KCl. The column was washed with 40 ml of Buffer C containing 0.1 M KCl and eluted with 150 ml of a linear gradient from 0.1 M to 0.8 M KCl in Buffer C. Fractions of 2.4 ml are collected. A profile is shown in Fig. 3. RNA polymerase I activity elutes in a sharp peak at 0.43 M KCl. Contaminant RNA polymerase III activity elutes at 0.7 M KCl. Active fractions are pooled and stored in liquid N_2. For long term storage the enzyme is concentrated by precipitation with ammonium sulfate and dissolved in Buffer C containing 50% glycerol.

Enzyme Purity

The enzyme obtained after sucrose gradient centrifugation is completely dependent on added DNA for activity and has a A_{260}/A_{280} ratio of 1.78. Direct analysis of DNA by the method of Burton (18) shows no detectable DNA (less than 15 μg of

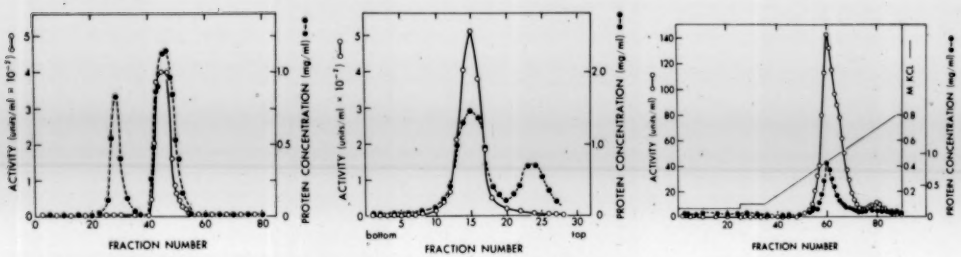

FIG. 1 (*left*). DEAE-Sephadex column chromatography of yeast RNA polymerase I. Fraction 3 was concentrated by ammonium sulfate precipitation and loaded onto a column (4 × 30 cm) of DEAE-Sephadex A-25 previously equilibrated as described in the text. Eighty fractions of 4.2 ml each were collected and assayed for protein and RNA polymerase activity.

FIG. 2 (*center*). Sucrose density gradient centrifugation of yeast RNA polymerase I. Gradients are prepared, loaded, and centrifuged as described in the text. Fractions of 0.5 ml were collected from the bottom of the tubes and assayed for protein and RNA polymerase activity.

FIG. 3 (*right*). DNA-cellulose column chromatography of yeast RNA polymerase I. Fractions from the sucrose gradients containing activity were pooled, diluted to 0.066 M KCl, and loaded onto a 25-ml column containing 7.5 mg of denatured calf thymus DNA. The column was washed with 1 column volume of Buffer C (see text) containing 0.1 M KCl and developed with a linear gradient containing column volumes of 0.1 M to 0.8 M KCl in Buffer C. Fractions 1 to 35 were of 4.8 ml; Fractions 36 to 90 were of 2.4 ml.

DNA/mg of protein). Thus the endogenous DNA present is less than 0.2% of the DNA added in the standard assay.

The purified RNA polymerase I is completely resistant to 40 μg/ml of α-amanitin. Thus it is free from enzyme II. This is confirmed by the subunit composition of the enzyme examined by polyacrylamide gel electrophoresis in sodium dodecyl sulfate, which shows the absence of the large molecular weight polypeptides of yeast polymerase II (26).

RNA polymerase III, as identified by its subunit structure (27) and elution behavior in DEAE-Sephadex column chromatography is present in variable amounts (3 to 5% of total activity) after sucrose gradient centrifugation and is removed in the DNA-cellulose step. Apparently homogeneous yeast RNA polymerase III can be removed in higher yield (10 to 20% of total activity) if the enzyme obtained after ion filtration is directly subjected to DNA-cellulose chromatography. The subunit structure of this enzyme has been determined by sodium dodecyl sulfate gel electrophoresis and will be reported elsewhere (27).

After the ion filtration step the purified enzyme is free from RNase activity as detected by using [^{3}H]RNA bound to filter paper discs and free of DNase as shown by the SV40 DNA allomorph conversion assay.

The purified enzyme was subjected to gel electrophoresis under nondenaturing conditions. Fig. 4 shows that native RNA polymerase I separates into two components in 5% acrylamide at pH 8.7. As will be shown below, analysis of the subunit composition of these two components indicates that they may correspond to different forms of the enzyme. The polymerase preparation behaves as a homogeneous protein by sedimentation velocity analysis. Fig. 5 shows the sedimentation behavior of the enzyme when centrifuged at 44,000 rpm at a concentration of 0.4 mg/ml. An $s_{20,w}$ value of 16.2 was obtained, which in the absence of data on the shape of the molecule is consistent with an approximate molecular weight of 650,000 ± 50,000 calculated from the molecular weights and molar ratios of the individual subunits (see below).

Subunit Structure

The subunit structure of the purified enzyme was examined by polyacrylamide gel electrophoresis in the presence of

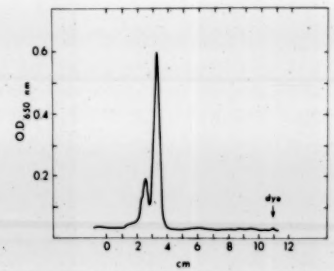

FIG. 4. Polyacrylamide gel electrophoresis of yeast RNA polymerases I under nondenaturing conditions. A sample of 10 μg of purified enzyme was subjected to electrophoresis on a 5% acrylamide slab gel as described under "Experimental Procedures."

sodium dodecyl sulfate. Fig. 6 shows the pattern of individual fractions from across the sucrose gradient and DNA-cellulose chromatography (Fig. 2 and 3) in 9% acrylamide gels. Nine protein bands are observed in all cases. The ratio of the amount of these polypeptides to enzyme activity is roughly constant. Scanning of each fraction shows no significant changes in the pattern except for subunit d which is present in slightly higher amounts in the first fractions of the DNA-cellulose peak. A more detailed analysis of the subunit structure is presented in Fig. 7 which shows the stained gel and scanning pattern of enzyme from pooled fractions of DNA-cellulose chromatography. Approximate molecular weights were calculated from the observed mobilities using protein standards of known molecular weights (Fig. 8). Protein bands appeared at 185,000 (a), 137,000 (b), 48,000 (c), 44,000 (d), 41,000 (e), 36,000 (f), 28,000 (g), 24,000 (h), 20,000 (i), 14,500 (j), and 12,000 (k). The same protein bands were obtained when purified enzyme was subjected to electrophoresis in 12% acrylamide gels (results not shown). In this instance, the polypeptides j (14,500) and k (12,000) resolve well from the tracking dye and are clearly evident. The molecular weights and the molar ratios are summarized in Table II. Preliminary information obtained from the mobility in gel electrophoresis in 8 M urea at pH 8.7[2]

[2] P. Valenzuela and F. Weinberg, unpublished results.

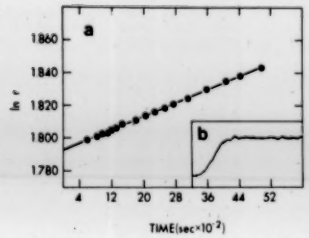

FIG. 5. Sedimentation velocity of purified yeast RNA polymerase I. Enzyme was sedimented in 0.05 M Tris-HCl, pH 7.9, 0.01 M $MgCl_2$, 0.2 M KCl, and 0.007 M 2-mercaptoethanol at a protein concentration of 0.4 mg/ml. *r* is the distance from the boundary to the center of rotation. *b* shows the boundary at 20.4×10^2 s.

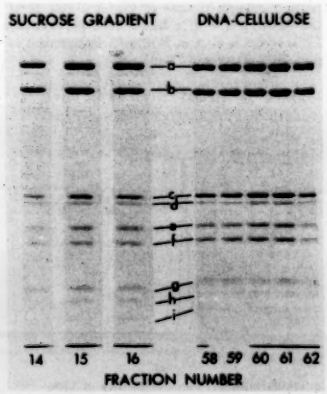

FIG. 6. Polyacrylamide sodium dodecyl sulfate gel electrophoresis patterns of yeast RNA polymerase I from peak tubes of the sucrose gradient of Fig. 2 and peak tubes of DNA-cellulose chromatography of Fig. 3. The 0.1% sodium dodecyl sulfate/9% acrylamide gels from sucrose gradient were loaded with 4 to 6 μg of protein/gel, gels from DNA-cellulose with 6 to 10 μg of protein/gel.

indicates that relative to each other, polypeptides a, b, c, and g are basic, i and k are acidic, and d, e, f, and j are of intermediate behavior.

Possible electrophoretic heterogeneity of yeast RNA polymerase I, suggested by the presence of two protein bands in native gel electrophoresis was further investigated by two-dimensional slab gel electrophoresis. A 5% acrylamide gel run under nondenaturing conditions in the first dimension, resolves the enzyme into two forms with slightly different electrophoretic mobility (Fig. 4). Electrophoresis under denaturing conditions in a second dimension was used to establish the subunit composition of each form. The results are shown in Fig. 9. The main component which migrates faster contains all the subunits except the polypeptides I_c and I_f. These two polypeptides are not found in the gels. They are more basic than the enzyme complex and presumably migrate toward the cathode in the conditions of the electrophoresis. The slower migrating component, which comprises 10 to 15% of the total protein, contains all the subunits. Although Fig. 9 does not show subunits I_j and I_k, the presence of these peptides in both forms of polymerase I was established by

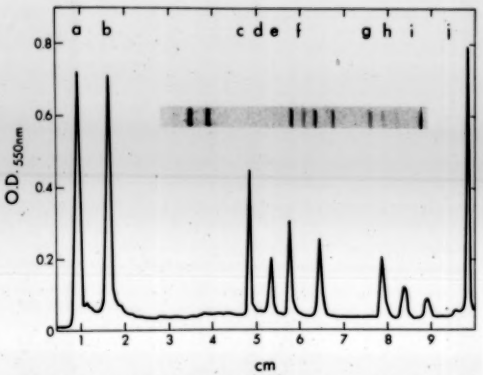

FIG. 7. Polyacrylamide sodium dodecyl sulfate gel electrophoresis pattern and corresponding densitometer tracing of yeast RNA polymerase I pooled after DNA-cellulose chromatography. 0.1% sodium dodecyl sulfate; 9% acrylamide; 6 μg of protein. Enzyme subunits are labeled *a* to *j* in order of decreasing molecular weights.

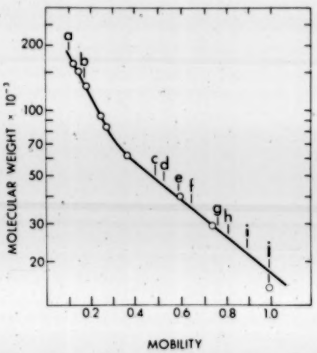

FIG. 8. Molecular weight estimation of yeast RNA polymerase I subunits. The migration of each subunit is indicated by a line and is relative to that of the tracking dye. The standard curve was made from the migration of proteins of known molecular weights (see text). Gels contain 9% acrylamide. *Lower case letters* refer to the same subunits of Fig. 7 and Table II.

using a 12% acrylamide gel in the second dimension (results not shown). Compared to enzyme from DNA-cellulose, the slow migrating component has slightly lower amounts of polypeptides I_c and I_f.

DISCUSSION

The procedure outlined above has eliminated all major constraints on the large scale preparation of yeast polymerase I without sacrificing yield or specific activity of the final product. The crucial features of this method are: (*a*) dilution of the extract or enzyme fractions is avoided, so the polymerase remains concentrated throughout the procedure; (*b*) no high speed centrifugation is necessary; (*c*) no cumbersome high resolution column chromatographic steps are employed; (*d*) no dialysis steps are necessary. Routinely 3.0 kg of cells can be processed in 5 days to obtain 30 to 45 mg of pure enzyme;

TABLE II

Subunit composition of yeast RNA polymerase I

Composition was examined by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate with enzyme obtained from DNA-cellulose chromatography. The 9% acrylamide gels were used except for the data of polypeptides j and k which were taken from 12% acryalmide gels. Molecular weights are averaged from three experiments from two different preparations. Molar ratios are normalized to polypeptide a. Molar ratios for polypeptides c, d, and f varied 10 to 30% in different preparations and the values shown correspond to those obtained from the data of Fig. 7.

Polypeptide	Molecular weight	Molar ratio
a	185,000 ± 10,000	1.00
b	137,000 ± 5,000	1.12
c	48,000 ± 2,000	2.00
d	44,000 ± 2,000	0.98
e	41,000 ± 2,000	1.50
f	36,000 ± 2,000	1.40
g	28,000 ± 1,000	1.92
h	24,000 ± 1,000	1.06
i	20,000 ± 1,000	1.06
j	14,500 ± 1,000	1.08
k	12,000 ± 1,000	

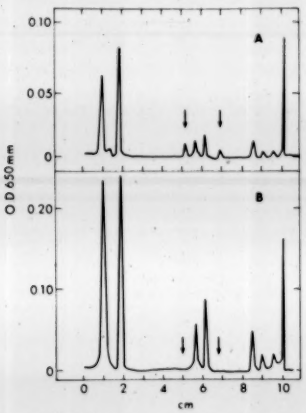

FIG. 9. Second dimension polyacrylamide sodium dodecyl sulfate gel electrophoresis of yeast RNA polymerase I. Gels contain 9% acrylamide and 0.1% sodium dodecyl sulfate. The first dimension 5% acrylamide native gels were loaded with 15 μg of protein. *A*, slower migrating component; *B*, faster migrating component. *Arrows* indicate the polypeptides I_c and I_f.

however, the method can be scaled up to process 10 kg or more of yeast so the purification of 0.1 or even 1 g quantities of enzyme is now feasible.

The first two steps involve chromatography in phosphocellulose and DEAE-cellulose, steps which have been extensively used in the purification of RNA polymerases (8). We have adopted a batchwise procedure similar to that employed by Ponta *et al.* (11) and Buhler *et al.* (15) for yeast and Mangel (28) for *Escherichia coli*, because it allows the processing of large amounts of starting material. The next step is ion filtration chromatography, a procedure devised by Kirkegaard *et al.* (16, 29), combining ion exchange and gel filtration in the same column. By adjusting the pH and ionic strength of the column it is possible to elute the purified enzyme after the excluded peak of noninteracting macromolecules and before the liquid column volume so that the enzyme is eluted in the sieving range. For yeast RNA polymerase I these conditions are met by equilibrating a DEAE-Sephadex column with 0.02 M Tris-HCl, pH 8.0, containing 0.1 M ammonium sulfate. The enzyme sample is introduced in the same buffer containing 0.35 M ammonium sulfate. Under these conditions nucleic acids interact stronger with the ion exchanger and are easily resolved from the enzyme. By this procedure a highly concentrated solution of enzyme can be processed in a short period of time (2 hours). This is an important advantage in the purification of RNA polymerases or other enzymes that are sensitive to dilution or dialysis for prolonged periods at 4°. The enzyme elutes in the sieving range with little dilution, and is easily concentrated for storage by ammonium sulfate precipitation. The salt concentration of the sample load can be as high as 0.35 M ammonium sulfate so that previous dialysis is avoided. The relatively high specific activity (195 units/mg) and the absence of significant endogenous template or RNase and DNase activities make the enzyme at this stage suitable for *in vitro* transcription studies.

The main contaminant removed in the sucrose gradient centrifugation step is a protein of molecular weight 160,000. This polypeptide, which co-purifies with the enzyme through the ion filtration step in roughly equimolar amounts with the large subunits, has a strong DNA binding ability and stimulates the activity of the purified enzyme especially at high ionic strength.[2]

The last step of the method is a chromatography on denatured calf thymus DNA-cellulose. This step does not significantly increase the specific activity of the enzyme, but is necessary to resolve RNA polymerase III and to remove other minor protein contaminants which co-purify with enzyme I through the first four steps. Enzyme III interacts very strongly with the DNA-cellulose and is eluted at 0.7 M KCl in a highly purified form.

Examination of the polypeptide composition of purified yeast RNA polymerase I reveals 10 to 11 putative subunits with molecular weights ranging from 185,000 to 12,000. The two large polypeptides (185,000 and 137,000) and some of the smaller polypeptides (48,000, 44,000, 28,000, 24,000, 20,000, and 14,500) are present in stoichiometric amounts. In addition there are polypeptides present in nonstoichiometric amounts (41,000 and 36,000). The molar ratios reported here cannot be taken as definitive. They are estimated from the amount of dye adsorbed, a method which may not be accurate (5). These polypeptides are termed subunits but we recognize there is no evidence that all of them are functionally involved in the RNA polymerase molecule. The very complex structure of the isolated enzyme suggests that some of the polypeptides may be regulatory components accompanying a basic polymerizing unit.

The subunit structure revealed through this work follows the general pattern reported by others for yeast RNA polymerase I (15, 30). However, there are some noticeable differences. Van Keulen *et al.* (30) reported, for the *Saccharomyces carlbergensis* enzyme, the presence of a subunit having a molecular weight of 54,000 and found no subunits in the regions 48,000, 41,000, 28,000, and 20,000. Buhler *et al.* (15) found no polypeptides with molecular weights 24,000 and 20,000 in their preparation. It seems likely that these disparities may arise from the different purification procedures and suggests the

existence of dissociable polypeptides which are not necessary for the polymerization reaction but may be required for another function.

Heterogeneity has been found in several eukaryotic RNA polymerases evidenced by chromatography or electrophoresis (26). Our results indicate that RNA polymerase I can be resolved by electrophoresis into two forms. The main component having all the subunits except the polypeptides I_e and I_f. This suggests that these subunits are more loosely bound than the others. Similar results were reported by Buhler *et al.* who reported that both forms are active (26, 31).

We have found that yeast RNA polymerases II[1] and/or III[2] also contain polypeptides with molecular weights 41,000, 28,000, 24,000, 20,000, and 14,500 which suggests that there is a common pool of small molecular weight subunits (27). Reconstitution of the enzyme from isolated subunits as well as peptide mapping studies on the small molecular weight polypeptides will be necessary to further elucidate the function, structure, and relationship between the various subunits of yeast RNA polymerases I, II, and III.

The general concepts and methods employed in this procedure may be useful in the preparation of RNA polymerases from other eukaryotic and prokaryotic sources.

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Phosphorylation of yeast RNA polymerases

DNA-DEPENDENT RNA polymerase activities in eukaryotic cells change during developmental[1,2] and physiological transitions[3–5]. Some changes occur rapidly and in the absence of protein synthesis. They presumably involve modulation of the activity of pre-existing RNA polymerase molecules rather than alterations of the rate of synthesis or degradation of these molecules. This modulation of polymerase activity could be a consequence of covalent modification of enzyme subunits, the rapid turnover of a protein factor required for catalysis, alteration of the template properties of the chromatin or some combination of these. The first possibility is attractive. The activity of many enzymes is controlled in this manner[9]. Cycles of phosphorylation and dephosphorylation regulate the activity of the enzymes involved in glycogen metabolism (reviewed in refs 9 and 10). Lee *et al.*[11] observed an increase in the specific activity of Rous sarcoma virus reverse transcriptase on incubation with protein kinase and ATP. Phosphorylation of β and β' subunits of *E. coli* RNA polymerase on infection by bacteriophage T7 has been implicated in the control of early transcription of T7 DNA[12]. Several groups[3,13,14] have proposed and presented preliminary evidence that phosphorylation of RNA polymerase is involved in regulation of polymerase activity in eukaryotes. Jungmann *et al.*[3] and Martelo and Hirsch[13] observed enhanced RNA polymerase activity when partially purified protein kinase, RNA

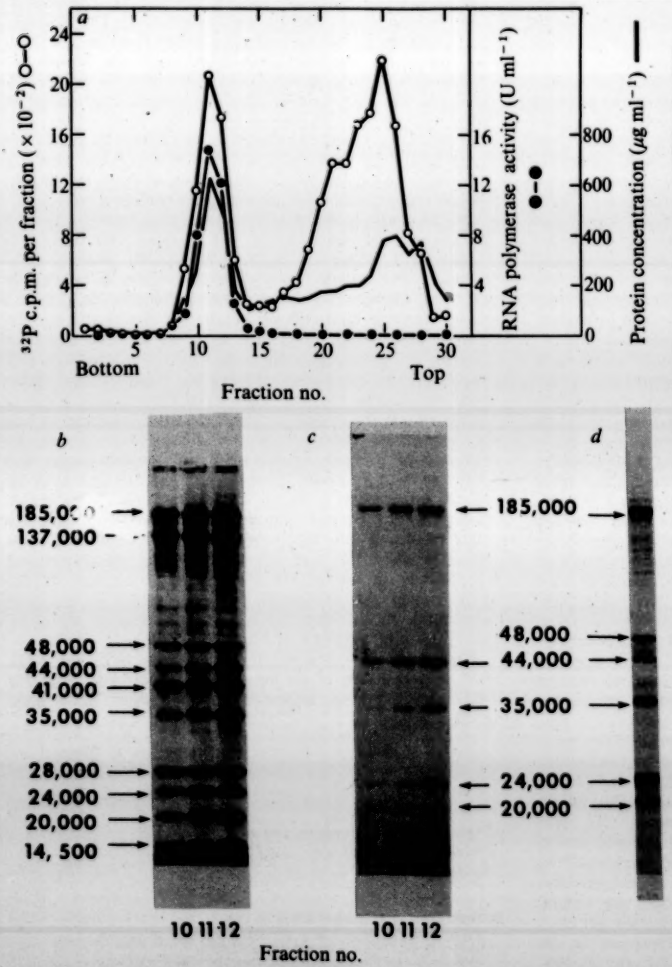

Fig. 1 Phosphorylation of yeast RNA polymerase I. ^{32}P-labelled RNA polymerase I was isolated from a prototrophic tetraploid strain of *Saccharomyces cerevisiae* (provided by Dr L. Hartwell) grown in complete medium (YEPD)[18] containing ^{32}P-phosphate (5 μCi ml^{-1}). Cells (2 l) were collected in the late-log phase of growth (30 g, wet weight) and spheroplasts were prepared by the two-step procedure of Cabib[19] as described by Wintersberger *et al.*[20]. RNA polymerase I was extracted in the presence of 1 mM ATP and was purified to fraction 3 by a micro-adaptation of Valenzuela *et al.*[21]. The ^{32}P-labelled enzyme fraction was mixed with unlabelled polymerase I (fraction 4)[21] and protein was precipitated by dialysis against a saturated ammonium sulphate solution. The precipitate was collected by centrifugation (10,000*g*, 20 min) and dissolved in 15% glycerol, 0.2 M KCl, 0.1 mM EDTA, 6 mM 2-mercaptoethanol, 50 mM Tris-HCl, *p*H 7.9. The RNA polymerase I was further purified by centrifugation in a 5–20% (w/v) sucrose gradient in 0.5 M KCl, 15% glycerol, 0.1 mM EDTA, 6 mM 2-mercaptoethanol, 50 mM Tris-HCl, *p*H 7.9 (SW56 rotor, 56,000 r.p.m., 15 h, 4 °C). Fractions were collected by tube puncture. *a*, Sucrose gradient profile of RNA polymerase I phosphorylated *in vivo*. RNA polymerase activity and protein concentration of each fraction were determined as before[21]. An aliquot of each gradient fraction was spotted on to a glass fibre filter (GF/C, Whatman). Filters were washed in 10% trichloroacetic acid (TCA). Contaminating ^{32}P-labelled nucleic acid was hydrolysed by boiling the filters in 5% TCA for 15 min. The filters were then washed twice more in 5% TCA, then in ethanol and dried. The ^{32}P-labelled protein was counted in Omnifluor (New England Nuclear)–toluene scintillant. *b*, Resolution of polypeptides by sodium dodecyl sulphate (SDS)–acrylamide gel electrophoresis of *in vivo* ^{32}P-labelled RNA polymerase. The polypeptides in each sucrose gradient fraction were resolved by 10% acrylamide slab–gel electrophoresis in the presence of 0.1% SDS[21] and stained with Coomassie blue. Buffers and solutions were prepared according to Laemmli[22]. The putative subunits of RNA polymerase I and assigned molecular weights are indicated by the arrows. *c*, Autoradiogram of RNA polymerase polypeptides labelled *in vivo* with ^{32}P. The stained gel prepared in (*b*) was dried and the ^{32}P-polypeptides were located by autoradiography. *d*, *In vitro* phosphorylation of yeast RNA polymerase I subunits. The standard reaction mixture contained in 0.050 ml: 10 μg of purified RNA polymerase I (21), 0.2 U of yeast protein kinase (1 U of protein kinase activity defined as 1 nmol of ^{32}P transferred from ^{32}P-ATP to 200 μg of phosvitin in 10 min at 30 °C), 60 mM Tris-HCl, *p*H 7.9, 5 mM $MgCl_2$, 6 mM NaF, 90 mM KCl, 5 mM 2-mercaptoethanol, 0.010 mM ^{32}P-ATP (specific activity, 1,000 d.p.m. pmol^{-1}) and 7.5% glycerol. After incubation for 15 min at 30 °C, the reaction was stopped by the addition of 0.005 ml of 5 mM ATP and subsequent transfer to 0 °C. Phosphorylated polymerase was precipitated from 10% TCA and the labelled polypeptides were identified after SDS–acrylamide gel electrophoresis and autoradiography as described above. No phosphorylation was observed in the absence of either polymerase or protein kinase.

polymerase and ATP were mixed. They observed the phosphorylation of proteins in their preparations and attributed the increase in polymerase activity to phosphorylation of the enzyme. They did not demonstrate phosphorylation of RNA polymerase polypeptides, and since the observed increase in RNA polymerase activity could also be a consequence of phosphorylation of contaminating acidic nuclear proteins which can stimulate polymerase activity[13–27], the cause of the enhanced polymerase activity is obscure. We report here the isolation of phosphorylated RNA polymerase I from yeast cells and the identification of the enzyme polypeptides phosphorylated *in vivo*. The same pattern of phosphorylation was obtained when highly purified RNA polymerase I was incubated with a yeast protein kinase preparation.

Yeast cells were grown in complete medium in the presence of ^{32}P-phosphate and collected in the late log phase of growth. RNA polymerase I was purified partially, mixed with unlabelled RNA polymerase I and subjected to sucrose gradient centrifugation. A significant fraction of the phosphorylated proteins sedimented with the enzymatic activity of the added RNA polymerase (Fig. 1*a*). The possible relationship of the phosphorylated polypeptides to the putative RNA polymerase I subunits was determined by resolution of the protein from the sucrose gradient fractions by polyacrylamide gel electrophoresis and subsequent autoradiography of the dried gel. Phosphorylated polypeptides migrated together with five of eleven polymerase I polypeptides (Fig. 1*b* and *c*). The 185,000, 44,000 35,000 and 24,000-dalton polypeptides were relatively more labelled than the 20.000-dalton polypeptide. There was also a small amount of radioactivity which migrated with the dye-front and may have been RNA. These data indicate that RNA polymerase is phosphorylated *in vivo* and is a phosphoprotein as isolated by common procedures.

Purified yeast RNA polymerases can be phosphorylated *in vitro* with a protein kinase which is purified together with RNA polymerase I but is resolved on sucrose gradient centrifugation. Whether this kinase is identical to those reported in yeast is unknown[23,24]. This enzyme uses either ATP or GTP as a phosphate donor, is not stimulated by cyclic AMP and will phosphorylate both acidic and basic phosphoprotein acceptors (G. I. B., unpublished results). The incubation of purified polymerase I and kinase resulted in the phosphorylation of the five polypeptides phosphorylated *in vivo* and, in addition, the 48,000-dalton polypeptide (Fig. 1*d*). The 185,000 and 24,000-dalton polypeptides were phosphorylated to a greater degree than the other polypeptides. The protein kinase also phosphorylated other molecules in the polymerase preparation. These phosphorylated non-polymerase polypeptides did not migrate together with stained polypeptides in the gel and it is not known whether they are a consequence of proteolysis of enzyme polypeptides or are contaminants of the polymerase preparation. Enzyme polypeptides of purified yeast RNA polymerases II and III were also phosphorylated by the protein kinase preparation (data not shown). The 33,500 and 24,000-dalton polypeptides of polymerase II and the 53,000, 24,000 and 20,000-dalton polypeptides of polymerase III were phosphorylated. (The isolation and subunit composition of polymerases II and III have been reported elsewhere[25,26].) Three of the phosphorylated polymerase I polypeptides (molecular weight 185,000; 44,000 and 35,000) are unique to polymerase I. Two-dimensional polyacrylamide gel electrophoresis showed the 24,000-dalton polypeptide to be common to RNA polymerases I, II and III; the 20,000-dalton polypeptide seems common to polymerases I and III (P.V., unpublished). The phosphorylation of the 24,000-dalton polypeptide of polymerases I, II and III and the 20,000-dalton polypeptide of polymerases I and III supports the contention that these are common subunits between the respective enzymes. Four of the phosphorylated polymerase I polypeptides (molecular weight 48,000 44,000, 35,000 and 24,000) are readily dissociated from the enzyme and seem to be important for activity. Polymerase I has been isolated that lacks the 48,000 and 44,000-dalton polypeptides[28] or the 48,000 and 35,000-dalton polypeptides[27] and has reduced enzymatic activity on double-stranded DNA templates. Polymerase .I lacking the 24,000-dalton polypeptide has also been isolated and is inactive on single- and double-stranded DNA and poly d(A-T) templates (P. V. and G. I. B., unpublished results).

In the phosphorylation experiments with purified polymerase I and protein kinase *in vitro*, only 0.5–1.0 mol of phosphate was bound to each mole of polymerase in optimal conditions. Since the data of the *in vivo* experiments indicate that there are at least five phosphorylation sites per enzyme, we presume that the purified polymerase is partially phosphorylated as isolated. The polypeptides of the purified polymerase molecules are phosphorylated to different extents in the *in vivo* and *in vitro* experiments. For example, the 44,000-dalton polypeptide is labelled more *in vivo* than *in vitro* while the reverse is found with the 24,000 and 20,000-dalton polypeptides. Thus there may be an equilibrium in the cell between phosphorylated and non-phosphorylated polymerase polypeptides. We observed that the 48,000-dalton peptide is phosphorylated only *in vitro*. One explanation for this result is that the enzyme molecule exists as a complex within the cell, such that the 48,000-dalton polypeptide is not accessible to protein kinase.

By modulating the charge density at a specific site in a polypeptide, phosphorylation and dephosphorylation could control the extent of interaction between enzyme polypeptides or between the enzyme and other molecules. In this way, phosphorylation of RNA polymerase could have a direct effect on transcription by altering the specific activity of the enzyme. It could also have an indirect effect on activity by modulating the association of polymerase with regulatory molecules or chromatin. Alternatively, phosphorylation may alter the turnover of the enzyme. We have begun to test the effects of phosphorylation on these aspects of polymerase function. In preliminary experiments, incubation of purified polymerase I with protein kinase and ATP or with bacterial alkaline phosphatase produced no effects on enzyme activity. These experiments are inconclusive so far because the isolated polymerase I is phosphorylated and the phosphatase may not remove the phosphate residues. We shall test the effects of phosphorylation on activity in standard assay conditions as well as the transcription of ribosomal genes in more physiological conditions, to elucidate the relationship between the phosphorylation of polymerase I and transcription.

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APPENDIX 3

Vol. 71, No. 1, 1976 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS

THE 24,000 DALTON SUBUNIT AND THE ACTIVITY OF YEAST RNA POLYMERASES

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SUMMARY: The subunits of RNA polymerase I are partially resolved during density gradient centrifugation. An analysis of the relative subunit composition with respect to specific catalytic activity shows that the molar ratio of the 24,000 dalton subunit directly correlates with polymerase activity. Since this polypeptide is found also in polymerases II and III, it may be required for activity of all yeast nuclear RNA polymerases.

INTRODUCTION

Yeast RNA polymerases I, II and III can be purified as complex structures composed of 10-11 different subunits (1-4). Knowledge of the function of the subunits is necessary to assess the role of RNA polymerases in specific transcription. It is of interest to know which of these polypeptides are strictly required for activity, which of them are regulatory proteins which modulate the activity of a "core" enzyme, and which of these are perhaps chromosomal or other proteins which copurify with the polymerase and may have ancillary functions.

We have previously reported that some of the putative subunits are uniquely associated with individual enzymes; on the other hand, the 28,000, 24,000 and 14,500 dalton subunits seem to be common to the three yeast polymerases (4). The availability of large amounts of pure yeast polymerase I (1) has allowed its use as a paradigm to test the function of its polypeptide components. Previous

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studies have shown that the isolated polymerase I complex is composed of polypeptides with approximate molecular weights 185,000, 137,000, 48,000, 44,000, 41,000, 36,000, 28,000, 24,000, 20,000, 14,500 and 12,000 (1,2). Some of these polypeptides are more loosely associated with the complex and can be removed during electrophoresis or chromatography in ion-exchange resins. Polypeptides of 48,000 and 36,000 daltons can be removed by acrylamide gel electrophoresis under non-dissociating conditions (1,2). Sentenac and collaborators first showed that an enzyme which lacks these two subunits is less active with native calf thymus DNA but is equally active with poly d(AT) template compared to the complete enzyme (2). Hager et al. (4) have purified RNA polymerase I to homogeneity without the 48,000 and 44,000 dalton polypeptides and have shown that the specific activity of this enzyme on native calf thymus template is half that of the complete enzyme. This report presents evidence suggesting that the 24,000 dalton subunit is requisite for yeast RNA polymerase I activity. Since this subunit is also present in polymerases II and III, it may be required for the activity of these enzymes as well.

MATERIALS AND METHODS

Yeast (Saccharomyces cerevisiae) RNA polymerase was purified to fraction 4 as previously described (1). Sucrose gradient centrifugation was performed as follows: 2 mg of polymerase I dissolved in 1 ml of buffer (0.05 M Tris-HCl, pH 8.0, 12.5% glycerol, 0.0001 M EDTA, 0.5 M KCl and 0.007 M 2-mercaptoethanol) was layered on a linear 5% to 20% (w/v) sucrose gradient made in the above buffer. The gradients were centrifuged for 28 hr at 40,000 rpm in a Beckman SW-41 rotor. Fractions were collected and assayed for protein concentration (5) and RNA polymerase activity on native and denatured calf thymus DNA and and poly d(AT) templates as previously described (1). Subunit analysis of gradient fractions was carried out in 0.1% SDS-12% acrylamide slab gels using the procedure of Laemmli (6) as described elsewhere (1).

RESULTS AND DISCUSSION

When yeast RNA polymerase I was subjected to sucrose gradient centrifugation under the conditions described above, the specific activity of the purified enzyme was not constant across the enzyme-protein-containing gradient fractions (Figure 1a). The same activity profile was observed using native

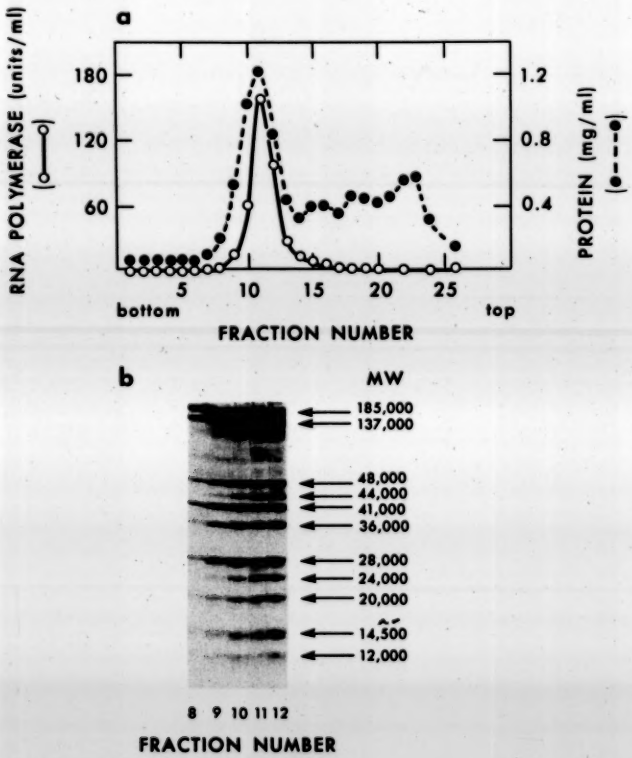

Figure 1. The subunit composition of yeast RNA polymerase I fractions from sucrose gradient centrifugation. (a) Fractions were collected and assayed for protein concentration (5) and enzyme activity on native calf thymus DNA. (b) Subunit analysis of the fractions.

and denatured calf thymus DNA and poly d(AT) templates. The gradient fractions were assayed by SDS gel electrophoresis. As shown in Figure 1b, the 44,000 and 24,000 dalton polypeptides are absent in the first fraction (fraction 8) and increase in relative amounts in the other fractions (9, 10, 11 and 12). The relative molar ratios of the polypeptides of all these fractions were determined by a densitometric analysis of the stained SDS gels. The results are shown in Figure 2. A chart correlating the molar ratio of each putative

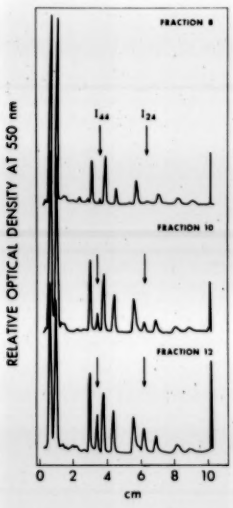

Figure 2. Densitometric tracing of yeast RNA polymerase I. Fractions correspond to those of the sucrose gradient described in Figure 1. Gel strips were scanned at 550 nm with a linear transport device attached to a Gilford spectrophotometer.

subunit (relative to the 137,000 dalton subunit) and the relative specific activity of each fraction of the gradient is shown in Figure 3. This analysis shows that the relative molar ratios of the 44,000 and 24,000 dalton polypep-

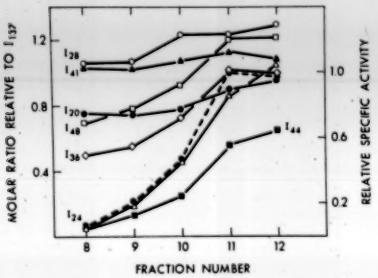

Figure 3. Correlation between the relative molar ratios of the subunits and the relative specific activity of yeast RNA polymerase I. The fractions correspond to those of the sucrose gradient described in Figure 1. Relative specific activity is designated by the dashed line.

tides increase sharply through the gradient fractions corresponding to the observed increase in specific activity of these polymerase fractions (dashed line). This relationship is essentially congruent in the case of the 24,000 dalton subunit. The results also show that the first fractions of the enzyme peak are deficient in the 48,000, 36,000 and 20,000 dalton polypeptides but their relative content does not correlate with the relative specific activity of the enzyme. The 41,000 and 28,000 dalton subunits are present at approximately constant levels in the various fractions.

Since it has been reported that the 48,000, 44,000 and 36,000 dalton polypeptides can be removed without appreciable changes in the activity of enzyme with a poly d(AT) template (2,4), our results suggest that the first fractions are inactive or less active because of the absence of the 24,000 dalton subunit. Therefore this polypeptide may be a basic component of the enzyme structure that is required for catalytic activity. A subunit of the same molecular weight is also present in yeast polymerase II and III (4) and it may also play a crucial role in the activity of these enzymes as well. Of course these experiments do not define the specific role of this polypeptide. It could be directly involved in catalysis, or in some ancillary

obligatory function of the enzyme. We have recently shown that the 24,000 dalton subunits of yeast polymerase I, II and III are phosphorylated by a yeast protein kinase (7) which suggests a possible role of phosphorylation of this polypeptide in the regulation of transcription in yeast. Reconstitution experiments may help to elucidate the function of this polypeptide.

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APPENDIX 4

Vol. 71, No. 4, 1976 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS

YEAST DNA DEPENDENT RNA POLYMERASES I, II AND III. THE EXISTENCE OF SUBUNITS COMMON TO THE THREE ENZYMES

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SUMMARY: The subunits of purified yeast RNA polymerases I, II and III have been analyzed by two-dimensional polyacrylamide gel electrophoretic subunit mapping techniques. The results suggest that polymerases I and III have two subunits in common, the 41,000 and 20,000 dalton peptides, which are not present in polymerase II . The 14,500 dalton peptide by all criteria is identical in polymerases I, II and III. The 28,000 and 24,000 subunits appear identical in polymerases I and II but have different charge properties in polymerase III.

INTRODUCTION

RNA polymerases I, II and III from eucaryotes are distinct enzymatic entities (1,2). Like the procaryotic enzyme each protein is characterized by two large unique subunits, but the eucaryotic counterparts have a much more complex array of smaller subunits (1,2). For example, yeast RNA polymerases I, II and III, as isolated, contain 8 to 13 polypeptides each (2,3). The differences in the molecular size of the various peptides suggest that the three polymerases are composed largely of products of different genes and hence are not interconvertible assemblies. However, the possibility that some of the peptides are very similar or identical has not been ruled out. Three polypeptides (28,000, 24,000, 14,500 daltons) have identical molecular weights in each of the three enzymes (2) and immunological cross-reaction between enzymes I and II has been reported (4). Furthermore, Thonart et al. have recently isolated a series of temperature-sensitive yeast mutants which are impaired in RNA synthesis (5). Mutations in any

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of three complementation groups affects the in vitro and in vivo activity of all three nuclear RNA polymerases. These results suggest the presence of three common subunits required for activity. Buhler et al. have already presented evidence suggesting the existence of three common subunits in polymerases I and II (6). We present here complementary studies of all three polymerases. The results suggest that polymerases I and III have two subunits in common which are not present in polymerase II (the 41,000 and 20,000 dalton peptides). In addition, the 14,500 dalton peptide by all criteria is identical in polymerases I, II and III. The 28,000 and 24,000 subunits appear identical in polymerases I and II but have different charge properties in polymerase III. Our results thus support the contention that some subunits are uniquely associated with each enzyme. Others are common between polymerases I and III, and I and II, still others are common between all three polymerases.

MATERIALS AND METHODS

Materials: Reagents, resins and other materials used are described in previous reports (2,3). Polymin P was obtained as a 50% solution in water from Gallard-Schlesinger, New York. A 5% stock solution was prepared as described by Burgess and Jendrisak (7).

Enzyme purification: Yeast (Saccharomyces cerevisiae) RNA polymerase I was purified as described previously (3). After removal of polymerase I by phosphocellulose (3), the extract was precipitated with Polymin P and polymerases II and III extracted with a buffer containing ammonium sulfate. The purification of polymerase II was continued by chromatography in DEAE-cellulose, phosphocellulose and sucrose gradient centrifugation. Polymerase III was purified by successive chromatographic steps in DEAE-cellulose, DEAE-Sephadex and DNA-cellulose. Enzymes I, II and III have specific activities (3) of 250, 450 and 150 units per mg of protein respectively. The detailed procedure for the purification of yeast polymerases II and III will be reported elsewhere (Valenzuela, P., Bell, G., Weinberg, F. & Rutter, W.J., manuscript in preparation). We thank G. Hager for a sample of polymerase II used in the initial phase of this research.

Gel electrophoresis: Sodium dodecyl sulfate (SDS)-acrylamide gel electrophoresis in one dimension was carried out by the method of Laemmli (8) as described previously (3). Two-dimensional subunit maps were performed in two systems. System a: 20 µg of each polymerase or a mixture of them were dissolved in 60 µl of a solution containing 8 M urea, 1 M 2-mercaptoethanol and 0.9 N acetic acid, heated in boiling water for 3 min and subjected to electrophoresis in 2.5 M urea - 10% acrylamide slabs as described by Panyim and Chalkley (9). For the second dimension, the corresponding strip of gel was equilibrated by stirring for 2 hr at room temperature in a solution containing 1% SDS, 2% 2-mercaptoethanol and 0.05 M Tris-HCl, pH 6.8 and layered on top of a polymerized 12% acrylamide gel slab containing 0.1% SDS (8). System b: electrophoresis in the first dimension was performed in 8 M urea, pH 8.6,

5% acrylamide gel slabs (11). For the second dimension, the corresponding strip of gel was equilibrated and electrophoresed as described in system a. After electrophoresis in the second dimension the gels were fixed, stained and destained as described previously (3). The position of each subunit spot in the map was determined by comparison with a standard sample of enzyme run parallel to the map in the second dimension.

Preparation of antiserum: Pure polymerase I (1 mg) was mixed with one volume of Freund's complete adjuvant and then injected into the hind toe pads of a rabbit. Two further injections of 1 mg in Freund's incomplete adjuvant were given 4 and 8 weeks later. Blood was collected by heart puncture 14 days after the third injection. The serum was separated from the clotted cells and stored at -20°C.

TABLE 1: Subunit Composition of Yeast RNA Polymerases I, II and III

Polymerase I MW $x10^{-3}$	Polymerase II MW $x10^{-3}$	Polymerase III MW $x10^{-3}$
185	170	160
137	145	128
		82
48		
44		
41		41
36		34
	33.5	
28	28	28
24	24	24
20		20
	18	
14.5	14.5	14.5
12.3	12.5	11

Enzymes were purified as described under Materials and Methods. Molecular weights were calculated from the electrophoretic mobilities ( ) as described previously (3). Polypeptides of 53,000, 40,500 and 37,000 daltons previously reported in polymerase III (2) do not occur in our present preparations and therefore presumably represent contaminants. The molar ratios of each subunit are reported elsewhere (2,3).

RESULTS AND DISCUSSION

The purified nuclear RNA polymerases from yeast have a complex subunit structure (2) (Table 1) and the majority of the subunits have a unique molecular weight. However, as indicated, each enzyme contains certain subunits of identical mass which suggests the existence of common subunits. Hildebrandt et al. (4) and Buhler et al. (6) have reported that yeast nuclear polymerases

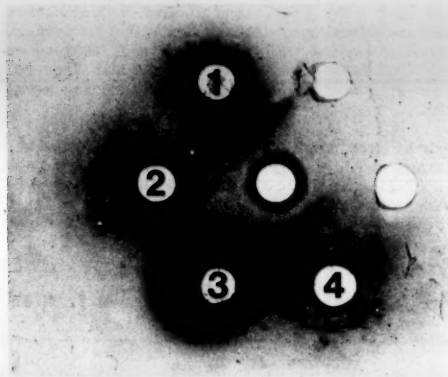

Figure 1. Double diffusion in agar. Immunodiffusion against rabbit antiserum was done at 4°C in 0.6% agar in 0.05 M sodium barbital, pH 8.2 and 0.2% sodium azide. The center well contained 5 µl of anti-polymerase I antiserum. Antigen wells contained: 1.5 µg of polymerase I (1); 2.5 µg of polymerase II (2); 3.5 µg of polymerase III (3); 4.2 µg of E. coli RNA polymerase (4). After formation of precipitin lines, the immunodiffusion plates were washed with 0.2% sodium bicarbonate overnight. The washed plates were then stained with 0.1% Coomassie brilliant blue in methanol:water:acetic acid (5:5:1) and destained in 10% acetic acid.

I and II share common antigenic determinants. Buhler et al. have presented evidence that the antigenic similarity between polymerase I and II is probably due to the presence of common small subunits since antibody prepared against subunit I_{185} did not cross-react with polymerase II (6). We have prepared antibodies to purified polymerase I. This antibody does not cross-react with polymerase II but does cross-react with polymerase III (Fig. 1) which suggests that polymerases I and III share common antigenic determinants which are lacking in polymerase II, in addition to those determinants shared between I and II. This observation suggests that there may be subunits common to polymerases I and III as well as between I and II.

We have also examined the question of common subunits by two-dimensional gel electrophoresis using systems similar to those that have been successfully used to resolve ribosomal proteins (10,12). Except for the two largest sub-

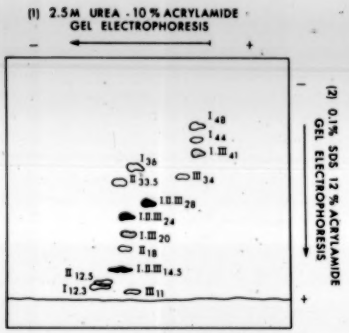

Figure 2. Schematic representation of an acid urea-SDS two-dimensional electrophoretogram of subunits of yeast RNA polymerases I, II and III. Solid spots represent co-migration of subunits of three enzymes; dashed spots represent co-migration of subunits of two enzymes; clear spots represent migration of subunits of an individual enzyme. Details in Materials and Methods.

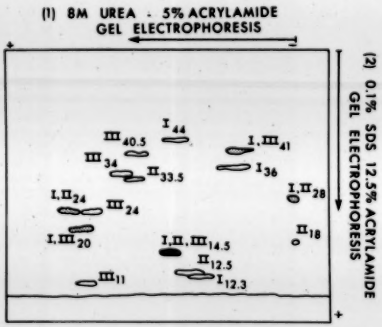

Figure 3. Schematic representation of basic urea-SDS two-dimensional electrophoretogram of subunits of yeast RNA polymerases I, II and III. Meaning of the intensity of the spots as indicated in Figure 2.

units, these systems effectively resolve the subunits of the enzymes. Figure 2 shows a schematic representation of the results obtained for polymerases I, II and III in the acid urea-SDS system. The first dimension, in 2.5 M urea and 0.9 N acetic acid, separates the polypeptides on the basis of the positive

charge density and the second dimension on the basis of molecular weight. The following subunits migrate together in this system: I_{41} and III_{41}; I_{28}, II_{28} and III_{28}; I_{24}, II_{24} and III_{24}; I_{20} and III_{20}; $I_{14.5}$, $II_{14.5}$ and $III_{14.5}$.

The electrophoretic mobility of the subunits was also examined in a second system designed to maximize the separation of the subunits by charge differences in the first dimension. The polypeptides were first separated by 5% acrylamide gel electrophoresis in the presence of 8 M urea, pH 8.8 and then subjected to 12.5% acrylamide gel electrophoresis in the presence of SDS. The results obtained are shown in Figure 3. The following subunits migrate together in this system: I_{41} and III_{41}; I_{28} and II_{28}; I_{24} and II_{24}; I_{20} and III_{20}; $I_{14.5}$, $II_{14.5}$ and $III_{14.5}$. Subunit III_{28} was not detected by this technique, presumably it is slightly more basic than I_{28} and II_{28} and migrates to the cathode in the first dimension. Subunit III_{24} was found slightly less acidic than I_{24} and II_{24}.

The data presented here do not allow a clear identification of the three polymerase genes described by Thonart et al. (5). However, there is a very likely possibility that these three genes correspond to subunits 28,000, 24,000 and 14,500 daltons. We have found that the 24,000 dalton peptide is susceptible to phosphorylation by yeast protein kinase (13), so that the difference in charge of III_{24} with respect to I_{24} and II_{24} may be due to a different degree of phosphorylation of an identical protein. Chemical modification (like acetylation or methylation) may also explain the different mobility of III_{28}. The report of Thonart et al. (5) also indicates that these three common subunits are required for catalytic activity in all three enzymes. The observation of Valenzuela et al. (14) who have isolated yeast polymerase I which lacks the 24,000 dalton subunit and is inactive on native and denatured DNA as well as poly (dA-dT) templates supports the idea of a catalytic role for this subunit in all three polymerases. Bell et al. (13) have demonstrated the in vivo and in vitro phosphorylation of the 24,000 dalton subunit of yeast polymerases I, II and III (also the 20,000 dalton subunit of polymerases I and III).

Their observations also support the hypothesis that the 24,000 dalton subunit is common to the three enzymes (and the 20,000 dalton subunit common to polymerases I and III). The phosphorylation of these common subunits also suggests that the polymerases may be regulated in the same manner under some circumstances.

Interestingly, mammalian RNA polymerases also have subunits of the same molecular weight: those of 25,000 and 16,500 daltons in calf thymus polymerases I and II and those of 29,000 and 19,000 daltons in polymerases I, II and III of murine plasmacytoma cells (1). All eucaryotic RNA polymerases may have a set of identical subunits which perform functions common to the three polymerases.

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